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POLYPEPTIDES HAVING BINDING AFFINITY FOR HER2.

Field of the invention

The present invention is related to a new polypeptide, which binds to Human Epidermal Growth Factor Receptor 2 (in the following referred to as HER2). The polypeptide is related to a domain of staphylococcal protein A (SPA) in that the sequence of the polypeptide corresponds to the sequence of the SPA domain having at least one substitution mutation. The present invention also relates to use of such a HER2 binding polypeptide as a medicament, more particularly use thereof for the preparation of a medicament for treatment of forms of cancer characterized by overexpression of HER2.

Background

Affibody® molecules

Molecules related to protein Z, derived from domain B of staphylococcal protein A (SPA) (Nilsson B *et al* (1987) Protein Engineering 1, 107-133), have been selected from a library of randomized such molecules using different interaction targets (see e g WO95/19374; WO00/63243; Nord K *et al* (1995) Prot Eng 8:601-608; Nord K *et al* (1997) Nature Biotechnology 15, 772-777). Different target molecules have been used to select such protein Z derivatives, e g as described in Nord K *et al* (1997, *supra*). The experiments described in this reference outline principles of the general technology of selecting protein Z derivatives against given targets, rather than being a study directed towards the express objective of obtaining a molecule with high enough affinity for use in a specific therapeutic or biotechnological application.

HER2 and its role in cancer diseases

The HER2 proto-oncogene encodes the production of a
185 kD cell surface receptor protein known as the HER2
protein or receptor (Hynes NE *et al* (1994) Biochim Bio-

5   phys Acta 1198:165-184). This gene is also sometimes re-
ferred to as neu, HER2/neu or c-erbB-2. Neu was first
discovered in rats that had been treated with ethylnitro-
sourea, and exhibited mutation of this gene (Shih C *et al*
(1981) Nature 290:261-264). The mutated version of neu

10   results in the production of a constitutively active form
of the receptor, and constitutes a potent oncogene that
can transform cells at low copy number (Hynes NE *et al*,
supra).

Normal cells express a small amount of HER2 protein

15   on their plasma membranes in a tissue-specific pattern.
No known ligand to HER2 has been elucidated; however,
HER2 has been shown to form heterodimers with HER1 (the
epidermal growth factor receptor, EGFR), HER3 and HER4 in
complex with the ligands for these receptors. Such forma-

20   tion of heterodimer leads to the activated HER2 receptor
transmitting growth signals from outside the cell to the
nucleus, thus controlling aspects of normal cell growth
and division (Sundaresan S *et al* (1999) Curr Oncol Rep
1:16-22).

25   In tumor cells, errors in the DNA replication system
may result in the existence of multiple copies of a gene
on a single chromosome, which is a phenomenon known as
gene amplification. Amplification of the HER2 gene leads
to an increased transcription of this gene. This elevates

30   HER2 mRNA levels and increases the concomitant synthesis
of HER2 protein, which results in HER2 protein overex-
pression on the surface of these tumor cells. This over-
expression can result in HER2 protein levels that are 10-
to 100-fold greater than those found in the adjacent nor-

35   mal cells. This, in turn, results in increased cell divi-
sion and a concomitantly higher rate of cell growth. Am-
plification of the HER2 gene is implicated in transforma-

tion of normal cells to the cancer phenotype (Hynes NE et al, supra; Sundaresan S et al, supra).

Overexpression of HER2 protein is thought to result in the formation of homodimers of HER2, which in turn re-
5   sults in a constitutively active receptor (Sliwkowski MX et al (1999) Semin Oncol 26(4 Suppl 12):60-70). Under these conditions, growth-promoting signals may be continuously transmitted into the cells in the absence of ligands. Consequently, multiple intracellular signal
10   transduction pathways become activated, resulting in unregulated cell growth and, in some instances, oncogenic transformation (Hynes NE et al, supra). Thus, the signal transduction mechanisms mediated by growth factor receptors are important targets for inhibiting cell replica-
15   tion and tumor growth.

Breast cancer is the most common malignancy among women in the United States, with 192200 new cases projected to have occurred in 2001 (Greenlee R et al (2001) CA Cancer J Clin 51:15-36). In approximately 25 % of all
20   breast cancer patients, there is an overexpression of the HER2 gene due to amplification thereof (Slamon DJ et al (1989) Science 244:707-712). This overexpression of HER2 protein correlates with several negative prognostic variables, including estrogen receptor-negative status, high
25   S-phase fraction, positive nodal status, mutated p53, and high nuclear grade (Sjogren S et al (1998) J Clin Oncol 16(2):462-469). According to Slamon et al (supra), the amplification of the HER2 gene was found to correlate strongly with shortened disease-free survival and short-
30   ened overall survival of node-positive patients.

For these reasons, it has been, and is still, an important goal to further pursue investigations into the role of HER2 in the pathogenesis and treatment of breast cancer. The identification of molecules that interact
35   with HER2 forms one part of this effort.

Preclinical in vitro studies have examined whether inhibition of HER2 activity could affect tumor cell

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growth. Treatment of SK-BR-3 breast cancer cells overex-
pressing HER2 protein with 4D5, one of several murine
anti-HER2 monoclonal antibodies, did indeed inhibit tumor
cell proliferation, compared to treatment with a control

5   monoclonal antibody. Administration of 4D5 to mice bear-
ing human breast and ovarian cancers (xenografts) that
overexpress HER2 prolonged their tumor-free survival
time. Similar studies demonstrated the growth inhibition
by anti-HER2 monoclonal antibodies in human gastric can-

10   cer xenografts in mice (Pietras RJ et al (1994) Oncogene
9:1829-1838).

Among the approaches to inhibiting the HER2 protein
abundantly present on tumor cell surfaces with an anti-
body, one therapy has become commercially available dur-

15   ing recent years. Thus, the monoclonal antibody 4D5, or
trastuzumab, is marketed for this purpose by F Hoffman-La
Roche and Genentech under the trade name of Herceptin®.

Notwithstanding the obvious advantages shown by an-
tibody therapy against cancers characterized by overex-

20   pression of HER2 protein, the fact remains that a variety
of factors have the potential of reducing antibody effi-
cacy (see e g Reilly RM et al (1995) Clin Pharmacokinet
28:126-142). These include the following: (1) limited
penetration of the antibody into a large solid tumor or

25   into vital regions such as the brain; (2) reduced ex-
travasation of antibodies into target sites owing to de-
creased vascular permeability; (3) cross-reactivity and
nonspecific binding of antibody to normal tissues, reduc-
ing the targeting effect; (4) heterogeneous tumor uptake

30   resulting in untreated zones; (5) increased metabolism of
injected antibodies, reducing therapeutic effects; and
(6) rapid formation of HAMA and human antihuman antibod-
ies, inactivating the therapeutic antibody.

In addition, toxic effects have been a major obsta-

35   cle in the development of therapeutic antibodies for can-
cer (Carter P (2001) Nat Rev Cancer 1:118-129; Goldenberg
DM (2002) J Nucl Med 43:693-713; Reichert JM (2002) Curr

Opin Mol Ther 4:110-118). Cross-reactivity with healthy
tissues can cause substantial side effects for unconju-
gated (naked) antibodies, which side effects may be en-
hanced upon conjugation of the antibodies with toxins or
5   radioisotopes. Immune-mediated complications include
dyspnea from pulmonary toxic effects, occasional central
and peripheral nervous system complications, and de-
creased liver and renal function. On occasion, unexpected
toxic complications can be seen, such as the cardiotoxic
10 effects associated with the HER-2 targeting antibody
trastuzumab (Herceptin®) (Schneider JW et al (2002) Semin
Oncol 29(3 suppl 11):22-28). Radioimmunotherapy with iso-
tope-conjugated antibodies also can cause bone marrow
suppression.
15   Despite the recent clinical and commercial success
of the currently used anticancer antibodies, a substan-
tial number of important questions thus remain concerning
the future of this therapeutic strategy. As a conse-
quence, the continued provision of agents with a compara-
20 ble affinity for HER2 remains a matter of substantial in-
terest within the field, as well as the provision of uses
of such molecules in the treatment of disease.

Disclosure of the invention
25   It is an object of the present invention to satisfy
this interest through the provision of a polypeptide that
is characterized by specific binding to HER2.
        A related object of the invention is an HER2 binding
polypeptide which exhibits little or no non-specific
30 binding.
        It is another object of the invention to provide an
HER2 binding polypeptide that can readily be used as a
moiety in a fusion polypeptide.
        Another object is the provision of an HER2 binding
35 polypeptide, which solve one or more of the known prob-
lems experienced with existing antibody reagents.

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A further object of the invention is to provide an
HER2 binding polypeptide, which is amenable to use in
therapeutic applications.

A related object is to find new forms for the treat-
5   ment, inhibition and/or targeting in the clinical setting
of cancer diseases characterized by an overexpression of
HER2 protein.

It is also an object to provide a molecule which can
be used as a reagent for the detection of HER2 at a low
10 concentration.

These and other objects are met by the different as-
pects of the invention as claimed in the appended claims.
Thus, in a first aspect, the invention provides a poly-
peptide, which has a binding affinity for HER2 and which
15 is related to a domain of staphylococcal protein A (SPA)
in that the sequence of the polypeptide corresponds to
the sequence of the SPA domain having from 1 to about 20
substitution mutations.

In an embodiment of the polypeptide according to
20 this aspect of the invention, the affinity thereof for
HER2 is such that the K_D value of the interaction is at
most 1×10^{-6} M. In another embodiment, the affinity of
the polypeptide for HER2 is such that the K_D value of the
interaction is at most 1×10^{-7} M.

25   In another embodiment, the polypeptide according to
the invention binds specifically to the extracellular do-
main, ECD, of the HER2 protein.

In accordance herewith, the present inventors have
found that it is possible to obtain a high-affinity HER2
30 binding polypeptide through substitution mutagenesis of a
domain from SPA, and that such a polypeptide is able to
interact with HER2. The inventive polypeptide finds ap-
plication as an alternative to antibodies against HER2 in
diverse applications. As non-limiting examples, it will
35 be useful in the treatment of cancers characterized by
HER2 overexpression, in inhibiting cell signaling by
binding to the HER2 on a cell surface, in the diagnosis

of cancer both *in vivo* and *in vitro*, in targeting of
agents to cells overexpressing HER2, in histochemical
methods for the detection of HER2, in methods of separa-
tion and other applications. The polypeptide according to
5   the invention may prove useful in any method which relies
on affinity for HER2 of a reagent. Thus, the polypeptide
may be used as a detection reagent, a capture reagent or
a separation reagent in such methods, but also as a
therapeutic agent in its own right or as a means for tar-
10   geting other therapeutic agents to the HER2 protein.
Methods that employ the polypeptide according to the in-
vention *in vitro* may be performed in different formats,
such as in microtiter plates, in protein arrays, on bio-
sensor surfaces, on tissue sections, and so on. Different
15   modifications of, and/or additions to, the polypeptide
according to the invention may be performed in order to
tailor the polypeptide to the specific use intended,
without departing from the scope of the present inven-
tion. Such modifications and additions are described in
20   more detail below, and may comprise additional amino ac-
ids comprised in the same polypeptide chain, or labels
and/or therapeutic agents that are chemically conjugated
or otherwise bound to the polypeptide according to the
invention. Furthermore, the invention also encompasses
25   fragments of the polypeptide that retain the capability
of binding to HER2.

        "Binding affinity for HER2" refers to a property of
a polypeptide which may be tested e g by the use of sur-
face plasmon resonance technology, such as in a Biacore®
30   instrument. HER2 binding affinity may be tested in an ex-
periment wherein HER2 is immobilized on a sensor chip of
the instrument, and a sample containing the polypeptide
to be tested is passed over the chip. Alternatively, the
polypeptide to be tested is immobilized on a sensor chip
35   of the instrument, and a sample containing HER2 is passed
over the chip. The skilled person may then interpret the
sensorgrams obtained to establish at least a qualitative

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measure of the polypeptide's binding affinity for HER2.
If a quantitative measure is sought, e g with the purpose
to establish a certain K_D value for the interaction, it
is again possible to use surface plasmon resonance meth-
5 ods. Binding values may e g be defined in a Biacore® 2000
instrument (Biacore AB). HER2 is immobilized on a sensor
chip of the instrument, and samples of the polypeptide
whose affinity is to be determined are prepared by serial
dilution and injected in random order. K_D values may then
10 be calculated from the results, using e g the 1:1 Lang-
muir binding model of the BIAevaluation 3.2 software pro-
vided by the instrument manufacturer.

As stated above, the sequence of the polypeptide ac-
cording to the present invention is related to the SPA
15 domain sequence in that from 1 to about 20 amino acid
residues of said SPA domain have been substituted for
other amino acid residues. However, the substitution mu-
tations introduced should not affect the basic structure
of the polypeptide. That is, the overall fold of the C_α
20 backbone of the polypeptide of the invention will be es-
sentially the same as that of the SPA domain to which it
is related, e g having the same elements of secondary
structure in the same order etc. Thus, polypeptides fall
under the definition of having the same fold as the SPA
25 domain if basic structural properties are shared, those
properties e g resulting in similar CD spectra. The
skilled person is aware of other parameters that are
relevant. This requirement of essentially conserving the
basic structure of the SPA domain, upon mutation thereof,
30 places restrictions on what positions of the domain may
be subject to substitution. When starting from the known
structure of the Z protein, for example, it is preferred
that amino acid residues located on the surface of the Z
protein are substituted, whereas amino acid residues bur-
35 ied within the core of the Z protein "three-helix bundle"
should be kept constant in order to preserve the struc-

tural properties of the molecule. The same reasoning ap-
plies to other SPA domains, and fragments thereof.

The invention also encompasses polypeptides in which
the HER2 binding polypeptide described above is present
5   as an HER2 binding domain, to which additional amino acid
residues have been added at either terminal. These addi-
tional amino acid residues may play a role in the binding
of HER2 by the polypeptide, but may equally well serve
other purposes, related for example to one or more of the
10   production, purification, stabilization, coupling or de-
tection of the polypeptide. Such additional amino acid
residues may comprise one or more amino acid residues
added for purposes of chemical coupling. An example of
this is the addition of a cysteine residue at the very
15   first or very last position in the polypeptide chain, i e
at the N or C terminus. Such additional amino acid resi-
dues may also comprise a "tag" for purification or detec-
tion of the polypeptide, such as a hexahistidyl (His$_6$)
tag, or a "myc" tag or a "flag" tag for interaction with
20   antibodies specific to the tag. The skilled person is
aware of other alternatives.

The "additional amino acid residues" discussed above
may also constitute one or more polypeptide domain(s)
with any desired function, such as the same binding func-
25   tion as the first, HER2-binding domain, or another bind-
ing function, or an enzymatic function, or a fluorescent
function, or mixtures thereof.

Thus, the invention encompasses multimers of the
polypeptide with affinity for HER2. It may be of inter-
30   est, e g when using the polypeptide according to the in-
vention for treatment of cancer or in a method of purifi-
cation of HER2, to obtain even stronger binding of HER2
than is possible with one polypeptide according to the
invention. In this case, the provision of a multimer,
35   such as a dimer, trimer or tetramer, of the polypeptide
may provide the necessary avidity effects. The multimer
may consist of a suitable number of polypeptides accord-

ing to the invention. These polypeptide domains according
to the invention, forming monomers in such a multimer,
may all have the same amino acid sequence, but it is
equally possible that they have different amino acid se-
5   quences. The linked polypeptide "units" in a multimer ac-
cording to the invention may be connected by covalent
coupling using known organic chemistry methods, or ex-
pressed as one or more fusion polypeptides in a system
for recombinant expression of polypeptides, or joined in
10   any other fashion, either directly or via a linker, for
example an amino acid linker.

Additionally, "heterogenic" fusion polypeptides, in
which the HER2 binding polypeptide constitutes a first
domain, or first moiety, and the second and further moie-
15   ties have other functions than binding HER2, are also
contemplated and fall within the ambit of the present in-
vention. The second and further moiety/moieties of the
fusion polypeptide may comprise a binding domain with af-
finity for another target molecule than HER2. Such a
20   binding domain may well also be related to an SPA domain
through substitution mutation at from 1 to about 20 posi-
tions thereof. The result is then a fusion polypeptide
having at least one HER2-binding domain and at least one
domain with affinity for said other target molecule, in
25   which both domains are related to an SPA domain. This
makes it possible to create multispecific reagents that
may be used in several biotechnological applications,
such as used as therapeutic agents or as capture, detec-
tion or separation reagents. The preparation of such mul-
30   tispecific multimers of SPA domain related polypeptides,
in which at least one polypeptide domain has affinity for
HER2, may be effected as described above for the multimer
of several HER2 binding "units". In other alternatives,
the second or further moiety or moieties may comprise an
35   unrelated, naturally occurring or recombinant, protein
(or a fragment thereof retaining the binding capability
of the naturally occurring or recombinant protein) having

a binding affinity for a target. An example of such a
binding protein, which has an affinity for human serum
albumin and may be used as fusion partner with the HER2
binding SPA domain derivative of the invention, is the
5 albumin binding domain of streptococcal protein G (SPG)
(Nygren P-Å et al (1988) Mol Recogn 1:69-74). A fusion
polypeptide between the HER2 binding SPA domain-related
polypeptide and the albumin binding domain of SPG thus
falls within the scope of the present invention. When the
10 polypeptide according to the invention is administered to
a human subject as a therapeutic agent or as a targeting
agent, the fusion thereof to a moiety which binds serum
albumin may prove beneficial, in that the half-life in
vivo of such a fusion protein may likely prove to be ex-
15 tended as compared to the half-life of the SPA domain re-
lated HER2 binding moiety in isolation (this principle
has been described e g in WO91/01743).

      Other possibilities for the creation of fusion poly-
peptides are also contemplated. Thus, the HER2 binding
20 SPA domain-related polypeptide according to the first as-
pect of the invention may be covalently coupled to a sec-
ond or further moiety or moieties, which in addition to,
or instead of, target binding exhibit other functions.
One example is a fusion between one or more HER2 binding
25 polypeptide(s) and an enzymatically active polypeptide
serving as a reporter or effector moiety. Examples of re-
porter enzymes, which may be coupled to the HER2 binding
polypeptide to form a fusion protein, are known to the
skilled person and include enzymes such as β-
30 galactosidase, alkaline phosphatase, horseradish peroxi-
dase, carboxypeptidase. Other options for the second and
further moiety or moieties of a fusion polypeptide ac-
cording to the invention include fluorescent polypep-
tides, such as green fluorescent protein, red fluorescent
35 protein, luciferase and variants thereof.

      Other options for the second and further moiety or
moieties of a fusion polypeptide according to the inven-

tion include a moiety or moieties for therapeutic appli-
cations. In therapeutic applications, other molecules may
also be coupled, covalently or non-covalently, to the in-
ventive polypeptide by other means. Non-limiting examples

5   include enzymes for "ADEPT" (antibody-directed enzyme
prodrug therapy) applications using the polypeptide ac-
cording to the invention for direction of the effector
enzyme (e g carboxypeptidase); proteins for recruitment
of effector cells and other components of the immune sys-

10 tem; cytokines, such as IL-2, IL-12, TNFα, IP-10; proco-
agulant factors, such as tissue factor, von Willebrand
factor; toxins, such as ricin A, *Pseudomonas* exotoxin,
calcheamicin, maytansinoid; toxic small molecules, such
as auristatin analogs, doxorubicin. Also, the above named

15 additional amino acids (notably hexahistidine tag, cys-
teine), provided with the aim of coupling chelators for
radioisotopes to the polypeptide sequence, are contem-
plated, in order to easily incorporate radiating nuclides
for diagnosis (e g ^{68}Ga, ^{76}Br, ^{111}In, ^{99}Tc, ^{124}I, ^{125}I) or

20 therapy (e g ^{90}Y, ^{131}I, ^{211}At).

The invention encompasses polypeptides in which the
HER2 binding polypeptide described above has been pro-
vided with a label group, such as at least one fluoro-
phore, biotin or a radioactive isotope, for example for

25 purposes of detection of the polypeptide.

With regard to the description above of fusion pro-
teins incorporating the HER2 binding polypeptide accord-
ing to the invention, it is to be noted that the designa-
tion of first, second and further moieties is made for

30 clarity reasons to distinguish between the HER2 binding
moiety or moieties on the one hand, and moieties exhibit-
ing other functions on the other hand. These designations
are not intended to refer to the actual order of the dif-
ferent domains in the polypeptide chain of the fusion

35 protein. Thus, for example, said first moiety may without
restriction appear at the N-terminal end, in the middle,
or at the C-terminal end of the fusion protein.

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An example of an SPA domain for use as a starting
point for the creation of a polypeptide according to the
invention is protein Z, derived from domain B of staphy-
lococcal protein A. As pointed out in the Background sec-
5   tion, this protein has previously been used as a scaffold
structure for the creation of molecules, denoted Affi-
body® molecules, capable of binding to a variety of tar-
gets. The 58 amino acid sequence of unmodified protein Z,
denoted Z_{wt}, is set out in SEQ ID NO:1 and illustrated in
10 Figure 1.

In an embodiment of the polypeptide according to the
invention, it is related to a domain of SPA in that the
sequence of the polypeptide corresponds to the sequence
of the SPA domain having from 4 to about 20 substitution
15 mutations. Other embodiments may have from 1 to about 13
substitution mutations, or from 4 to about 13 substitu-
tion mutations.

In a more specific embodiment of the polypeptide ac-
cording to the invention, its sequence corresponds to the
20 sequence set forth in SEQ ID NO:1 having from 1 to about
20 substitution mutations, such as from 4 to about 20,
from 1 to about 13 or from 4 to about 13 substitution mu-
tations.

The polypeptide according to the invention may in
25 some embodiments correspond to the sequence set forth in
SEQ ID NO:1, which sequence comprises substitution muta-
tions at one or more of the positions 13, 14, 28, 32 and
35. Additionally, the sequence of the polypeptide accord-
ing to the invention may comprise substitution mutations
30 at one or more of the positions 9, 10, 11, 17, 18, 24, 25
and 27.

The sequence of a polypeptide according to another
embodiment of the invention corresponds to SEQ ID NO:1,
comprising at least a substitution mutation at position
35 13 from phenylalanine to tyrosine.

The sequence of a polypeptide according to another
embodiment of the invention corresponds to SEQ ID NO:1,

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comprising at least a substitution mutation at position 14 from tyrosine to tryptophan.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 28 from asparagine to an amino acid residue selected from arginine and histidine, more preferably to arginine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 32 from glutamine to arginine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 35 from lysine to tyrosine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 10 from glutamine to arginine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 11 from asparagine to threonine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 17 from leucine to valine.

The sequence of a polypeptide according to another embodiment of the invention corresponds to SEQ ID NO:1, comprising at least a substitution mutation at position 27 from arginine to an amino acid residue selected from lysine and serine.

A preferred polypeptide according to the invention corresponds to SEQ ID NO:1, comprising at least the fol-lowing mutations: F13Y, Y14W, N28R, Q32R and K35Y.

Examples of specific sequences of different embodi-ments of the polypeptide according to the invention, each

comprising one or more of the specific mutations de-
scribed above, are set out in SEQ ID NO:2-79 and illus-
trated in Figure 1. HER2 binding characteristics of these
polypeptides are disclosed in the examples that follow
5   this general description of the invention.

As an alternative to using the unmodified SPA do-
main, the SPA domain may also be subjected to mutagenesis
in order to increase the stability thereof in alkaline
conditions. Such stabilization involves the site-directed
10   substitution of any asparagine residues appearing in the
unmodified sequence with amino acid residues that are
less sensitive to alkaline conditions. When using the
polypeptide according to the invention as an affinity
ligand in affinity chromatography, this property of hav-
15   ing a reduced sensitivity to alkali provides benefits;
affinity chromatography columns are frequently subjected
to harsh alkali treatment for cleaning in place (CIP) be-
tween separation runs, and the ability to withstand such
treatment prolongs the useful lifetime of the affinity
20   chromatography matrix. As an example, making use of pro-
tein Z as starting point, the polypeptide according to
the invention may, in addition to the substitution muta-
tions conferring HER2 binding, have modifications in that
at least one asparagine residue selected from N3, N6,
25   N11, N21, N23, N28, N43 and N52 has been substituted with
an amino acid residue that is less sensitive to alkaline
treatment. Non-limiting examples of such polypeptides are
those having the following sets of mutations (with re-
spect to the sequence of Z_{wt}): N3A; N6D; N3A, N6D and
30   N23T; N3A, N6D, N23T and N28A; N23T; N23T and N43E; N28A;
N6A; N11S; N11S and N23T; N6A and N23T. Thus, these SPA
domains, as well as other SPA domains that have been sub-
jected to asparagine mutation for stability reasons, may
all be subjected to further substitution mutation of
35   amino acid residues in order to obtain the HER2 binding
polypeptide of the invention. Alternatively, an HER2
binding polypeptide of the invention which comprises as-

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paragine residues may be subjected to further mutation to
replace such residues. Evidently, this latter alternative
is only possible to the extent that the HER2 binding ca-
pability of such a molecule is retained.

5   The invention also encompasses polypeptides that
have been derived from any of the polypeptides described
above, through generation of a fragment of the above
polypeptides, which fragment retains HER2 affinity. The
fragment polypeptide is such that it remains stable, and

10   retains the specificity to bind HER2. The possibility to
create fragments of a wild-type SPA domain with retained
binding specificity to immunoglobulin G is shown by Bra-
isted AC and Wells JA et al in Proc Natl Acad Sci USA
93:5688-5692 (1996). By using a structure-based design

15   and phage display methods, the binding domain of a three-
helix bundle of 59 residues was reduced to a resulting
two-helix derivative of 33 residues. This was achieved by
stepwise selection of random mutations from different re-
gions, which caused the stability and binding affinity to

20   be iteratively improved. Following the same reasoning
with the polypeptides according to the first aspect of
the invention, the skilled man would be able to obtain a
"minimized" HER2 binding polypeptide with the same bind-
ing properties as that of the "parent" HER2 polypeptide.

25   Hence, a polypeptide constituting a fragment of a poly-
peptide according to the above aspect of the invention,
which fragment retains binding affinity for HER2, is a
further aspect of the invention.

         Another aspect of the present invention relates to a
30   nucleic acid molecule comprising a sequence which encodes
a polypeptide according to the invention.

         A further aspect of the present invention relates to
an expression vector comprising the nucleic acid molecule
of the previous aspect, and other nucleic acid elements

35   that enable production of the polypeptide according to
the invention through expression of the nucleic acid
molecule.

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Yet another aspect of the present invention relates to a host cell comprising the expression vector of the previous aspect.

The latter three aspects of the invention are tools
5   for the production of a polypeptide according to the invention, and the skilled person will be able to obtain them and put them into practical use without undue burden, given the information herein concerning the polypeptide that is to be expressed and given the current level
10 of skill in the art of recombinant expression of proteins. As an example, a plasmid for the expression of unmodified protein Z (see e g Nilsson B et al (1987), supra) may be used as starting material. The desired substitution mutations may be introduced into this plasmid,
15 using known techniques, to obtain an expression vector in accordance with the invention.

However, the polypeptide according to the invention may also be produced by other known means, including chemical synthesis or expression in different prokaryotic
20 or eukaryotic hosts, including plants and transgenic animals. When using chemical polypeptide synthesis, any of the naturally occurring amino acid residues in the polypeptide as described above may be replaced with any corresponding, non-naturally occurring amino acid residue or
25 derivative thereof, as long as the HER2 binding capacity of the polypeptide is not substantially compromised. The binding capability should at least be retained, but replacement with a corresponding, non-naturally occurring amino acid residue or derivative thereof may actually
30 also serve to improve the HER2 binding capacity of the polypeptide. Also, the incorporation of a non-naturally occurring amino acid may be performed in order to provide a site for alternative coupling of molecules (e g labels, effectors, chelators etc) to the HER2 binding polypep-
35 tide. Non-classical amino acids, or synthetic amino acid analogs, include, but are not limited to, the D-isomers of the common amino acids, α-amino isobutyric acid, 4-

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amino butyric acid, 2-amino butyric acid, 6-amino hexa-
noic acid, 2-amino isobutyric acid, 3-amino propionic
acid, ornithine, norleucine, norvaline, hydroxyproline,
sarcosine, citrulline, cysteic acid, t-butylglycine, t-
5 butylalanine, phenylglycine, cyclohexylalanine, β-
alanine, fluoroamino acids, designer amino acids such as
β-methyl amino acids, Cα-methyl amino acids, Nα-methyl
amino acids, and amino acid analogs in general. Further-
more, the amino acid residues can be present in D or L
10 form.

The present invention also concerns different as-
pects of using the above-described HER2 binding polypep-
tide, as well as various methods for treatment, diagnosis
and detection in which the polypeptide is useful due to
15 its binding characteristics. When referring to the "HER2
binding polypeptide" in the following description of
these uses and methods, this term is intended to encom-
pass the HER2 binding polypeptide alone, but also all
those molecules based on this polypeptide described above
20 that e g constitute fragments thereof and/or incorporate
the HER2 binding polypeptide as a moiety in a fusion pro-
tein and/or are conjugated to a label or therapeutic
agent and/or are provided with additional amino acid
residues as a tag or for other purposes. As explained
25 above, such fusion proteins, derivatives, fragments etc
form a part of the present invention.

Thus, in one such aspect, the invention provides use
of the HER2 binding polypeptide as described herein as a
medicament.
30   In a further aspect, the invention provides use of
the HER2 binding polypeptide as described herein in the
preparation of a medicament for the treatment of at least
one form of cancer characterized by overexpression of
HER2. One particular form of cancer characterized by
35 overexpression of HER2 is a breast cancer. As described
in the Background section, approximately 25 % of all

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breast cancer patients show an overexpression of HER2
(Slamon DJ et al, supra).

    Without wishing to be bound by this theory, the
polypeptide described herein is thought to be useful as a
5   therapeutic agent based on at least one of the following
mechanisms: (i) Potentiation of chemotherapy (cytotoxic),
in that administration of the polypeptide will function
in synergy with existing and coming chemotherapies and
hormonal therapies. Blocking of the HER2 protein on cell
10 surfaces has been shown to prevent DNA repair following
the impact of DNA-damaging drugs (Pietras RJ et al (1994)
Oncogene 9:1829-1838). (ii) Inhibition of the prolifera-
tion of tumor cells (cytostatic). This reasoning is based
on the observation that downregulation of HER2 protein
15 occurs when a molecule (antibody) attaches to the HER2
protein on the cell surface, causing some receptors to be
endocytosed, limiting the signal for further cell growth
(Baselga J et al (1998) Cancer Res 58:2825-2831; Sli-
wkowski MX et al, supra).
20   A related aspect of the present invention is the
provision of a method for the treatment of at least one
form of cancer characterized by overexpression of HER2,
which method comprises administering to a subject in need
of such treatment a therapeutically effective amount of a
25 composition, which comprises a HER2 binding polypeptide
as described herein as an active substance.
    The HER2 binding properties of the polypeptide ac-
cording to the invention, together with the suitability
of the polypeptide for the creation of fusion proteins
30 and/or labeled binding molecules, means that the polypep-
tide may also be useful for targeting of other active
substances to the site of a tumor which comprises cells
that overexpress HER2. Thus, another aspect of the pre-
sent invention is the provision of use of the HER2 bind-
35 ing polypeptide as described herein in conjugated to an
substance with anti-cancer activity for delivery of said
substance to cells that overexpress HER2. The conjugated

substance may also be one that functions to elicit a re-
sponse of the subject's endogenous immune system. Natural
killer (NK) cells, or other effectors of the immune sys-
tem, may be attracted to the complex of HER2 and HER2
5   binding polypeptide on the cell's surface through the
provision of a fusion moiety that serves the function of
recruiting such effectors. The NK cells or other effec-
tors, having detected that the cell is abnormal, attach
to the HER2 binding fusion protein. Eventually, the can-
10   cer cell is consumed by the NK cells (Sliwkowski MX *et
al, supra*; Pegram MD *et al* (1997) Proc Am Assoc Cancer
Res 38:602, Abstract 4044).

        Such an active substance may be a protein coupled to
the HER2 binding polypeptide by fusion or by chemical
15   linkage, such as chosen among effector enzymes for
"ADEPT" (antibody-directed enzyme prodrug therapy) appli-
cations; proteins for recruitment of effector cells and
other components of the immune system; cytokines, such as
IL-2, IL-12, TNFα, IP-10; procoagulant factors, such as
20   tissue factor, von Willebrand factor; toxins, such as
ricin A, *Pseudomonas* endotoxin, calcheamicin, maytansi-
noid. Alternatively, the active substance may be a cyto-
toxic drug, such as auristatin analogs or doxorubicin, or
a radioactive isotope (e g ^{90}Y, ^{131}I, ^{211}At), which isotope
25   may be associated with the HER2 binding polypeptide di-
rectly, or associated via a chelating agent, such as the
well known chelators DOTA or DTPA.

        In a related aspect, the invention also provides a
method of directing a substance having an anti-cancer ac-
30   tivity to cells overexpressing HER2 *in vivo*, comprising
administering a conjugate of said active substance and a
HER2 binding polypeptide as described herein to a pa-
tient. The conjugate is suitably as described in the pre-
ceding paragraph.

35   Another aspect of the present invention is the use
of the HER2 binding polypeptide as described herein for
the detection of HER2 in a sample. For example, such de-

tection may be performed with the aim of diagnosing dis-
ease states characterized by overexpression of HER2. The
detection of HER2 presence in a sample may be performed
in vitro or in vivo. A preferred option for in vivo diag-
5   nosis is the use of positron emission tomography, PET.
The sample in question may e g be a biological fluid sam-
ple or a tissue sample. A common method, in use today
with antibodies directed against HER2, which method may
be adapted for use with the HER2 binding polypeptide of
10 the present invention, is histochemical detection of HER2
presence used for identification of HER2 protein overex-
pression in fresh, frozen, or formalin-fixed, paraffin-
embedded tissue samples. For the purposes of HER2 detec-
tion, the polypeptide according to the invention may
15 again be used as part of a fusion protein, in which the
other domain is a reporter enzyme or fluorescent enzyme.
Alternatively, it may be labeled with one or more fluo-
rescent agent(s) and/or radioactive isotope(s), option-
ally via a chelator. Suitable radioactive isotopes in-
20 clude ^{68}Ga, ^{76}Br, ^{111}In, ^{99}Tc, ^{124}I and ^{125}I.

Yet another aspect of the present invention is con-
stituted by the use of an HER2 binding polypeptide as de-
scribed herein in a method of detecting HER2 in a bio-
logical fluid sample. This method comprises the steps of
25 (i) providing a biological fluid sample from a patient to
be tested, (ii) applying an HER2 binding polypeptide as
described herein to the sample under conditions such that
binding of the polypeptide to any HER2 present in the
sample is enabled, (iii) removing non-bound polypeptide,
30 and (iv) detecting bound polypeptide. The amount of the
detected bound polypeptide is correlated to the amount of
HER2 present in the sample. In step (ii), the application
of HER2 binding polypeptide to the sample may be per-
formed in any suitable format, and includes for example
35 the situation when the HER2 binding polypeptide is immo-
bilized on a solid support with which the sample is

brought into contact, as well as set-ups in which the
HER2 binding polypeptide is present in solution.

Another, related, aspect of the present invention is
a method for the detection of HER2 in a sample, compris-
5   ing the steps of (i) providing a tissue sample suspected
of containing HER2, for example a cryostat section or a
paraffin-embedded section of tissue, (ii) applying an
HER2 binding polypeptide according to the invention to
said sample under conditions conducive for binding of the
10   polypeptide to any HER2 present in the sample, (iii) re-
moving non-bound polypeptide, and (iv) detecting bound
polypeptide. The amount of the detected bound polypeptide
is correlated to the amount of HER2 present in the sam-
ple.

15   Also provided by the present invention is a kit for
diagnosis of HER2 overexpression in a tissue sample, com-
prising the HER2 binding polypeptide according to the in-
vention fused to a reporter enzyme (such as alkaline
phosphatase or horseradish peroxidase), reagents for de-
20   tection of enzyme activity, and positive and negative
control tissue slides.

Also provided by the present invention is a kit for
diagnosis of HER2 overexpression in a tissue sample, com-
prising the HER2 binding polypeptide according to the in-
25   vention fused to a tag for detection by an antibody (such
as a flag tag or myc tag), a primary antibody specific
for the tag, a secondary antibody specific for the pri-
mary antibody and conjugated to a reporter enzyme, re-
agents for detection of enzyme activity, and positive and
30   negative control tissue slides.

One area in diagnosis applications is the *in vivo*
detection of cancer cells or aggregates thereof. The in-
vention provides a kit for performing such diagnosis,
which kit comprises the HER2 binding polypeptide accord-
35   ing to the invention labeled with a chelator, a diagnos-
tic radioactive isotope (non-limiting examples are ^{68}Ga,

^{76}Br, ^{111}In, ^{99}Tc, ^{124}I and ^{125}I), and reagents for the
analysis of the incorporation efficiency.

As described above, the invention encompasses use of
the HER2 binding polypeptide according to the invention
5   to target active substances to cells that overexpress
HER2, such as certain types of cancer cells. The inven-
tion also provides a kit for this purpose, comprising
HER2 binding polypeptide according to the invention la-
beled with a chelator, a therapeutic radioactive isotope
10 (non-limiting examples are ^{90}Y, ^{131}I, ^{211}At), and reagents
for the analysis of the incorporation efficiency.

Brief description of the drawings

Figure 1 shows an alignment of the sequences of the
15 sequence listing. The amino acid positions that have been
subjected to modification in the polypeptides Z_{HER2} ac-
cording to the invention (represented by SEQ ID NO:2-79)
are indicated in bold.

Figure 2 is a schematic illustration of the amino
20 acid sequence of a fusion polypeptide produced in Example
1. Z_{HER2} represents a HER2 binding domain with a sequence
selected from SEQ ID NO:2-3 and His$_6$ represents a hexa-
histidyl tag.

Figure 3 shows the result of gel electrophoresis of
25 purified fusion proteins. Lane 1: His$_6$-$Z_{HER2\ A}$ (8.7 kDa);
Lane 2: His$_6$-$Z_{HER2\ B}$ (8.7 kDa); M: Molecular weight marker
(LMW-SDS Marker Kit, Amersham Biosciences #17-0446-01).

Figure 4 shows Biacore sensorgrams obtained after
injection of 10 μM of the His$_6$-$Z_{HER2\ A}$ fusion protein over
30 sensor chip surfaces having A: HER2, B: HIV-1 gp120, and
C: BB immobilized thereto.

Figure 5 shows Biacore sensorgrams obtained after
injection of 10 μM of the His$_6$-$Z_{HER2\ B}$ fusion protein over
sensor chip surfaces having A: HER2, B: HIV-1 gp120, and
35 C: BB immobilized thereto.

Figure 6 shows Biacore sensorgrams obtained after
injection of A: 1 μM; B: 2 μM; C: 5 μM; D: 10 μM, E: 20

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μM; F: 40 μM of the His_6-$Z_{HER2\ A}$ fusion protein over a sen-
sor chip surface having HER2 immobilized thereto.

Figure 7 shows Biacore sensorgrams obtained after
injection of A: 1 μM; B: 2 μM; C: 5 μM; D: 10 μM, E: 20
5   μM; F: 40 μM of the His_6-$Z_{HER2\ B}$ fusion protein over a sen-
sor chip surface having HER2 immobilized thereto.

Figure 8A shows Biacore sensorgrams obtained after
injection of His_6-$Z_{HER2\ A}$ over a HER2-ECD flow-cell surface
at selected concentrations; 312.5 nM (filled diamonds),
10   156.3 nM (filled circles), 78.2 nM (filled triangles),
39.1 nM (open squares), 19.6 nM (open diamonds), and 9.8
nM (open circles).

Figure 8B shows Biacore sensorgrams obtained after
injection of His_6-$Z_{HER2\ B}$ over a HER2-ECD flow-cell surface
15   at selected concentrations; 625 nM (filled squares),
312.5 nM (filled diamonds), 156.3 nM (filled circles),
78.2 nM (filled triangles), 39.1 nM (open squares), and
19.6 nM (open diamonds).

Figure 9 shows specificity of the His_6-$Z_{HER2\ A}$ binding
20   to SKBR-3 cells. ^{125}I-labelled His_6-$Z_{HER2\ A}$ was allowed to
bind to SKBR-3 cells with an estimated theoretical
ligand:HER2 receptor ratio of 5:1. Values are means of
three measurements. Error bars represent standard devia-
tions.

25   Figure 10 shows Biacore sensorgrams obtained after
injection of purified His_6-$Z_{HER2\ A}$ (open squares) and His_6-
$(Z_{HER2\ A})_2$ (filled squares) over a sensor chip flow-cell
surface containing amine-coupled HER2-ECD. The y values
of the curves have been normalized to between 0 and 100
30   Resonance Units. The inserted SDS-PAGE gel (Tris-Glycine
16% homogenous gel, reducing conditions) shows the ex-
pressed and IMAC-purified His_6-$Z_{HER2\ A}$ (lane 1) and His_6-
$(Z_{HER2\ A})_2$ (lane 2). Lane M, marker proteins with molecular
masses in kilodaltons.

35   Figure 11 shows a comparison of biodistribution of
radioactivity in tumor bearing nude mice 1 h after injec-
tion of ^{125}I-benzoate-$(Z_{HER2\ A})_2$. Blocked: Data for mice

25

pre-injected with non-labeled $(Z_{HER2 A})_2$. Non-blocked: Data
for mice without pre-injection.

Figure 12 shows a comparison of biodistribution of
radioactivity in tumor bearing nude mice 4 h after injec-
5   tion of ^{125}I-benzoate-$(Z_{HER2 A})_2$ (Z4dimer) or ^{125}I-benzoate-
Z_{Taq} (Ztaq4:5).

Figure 13 shows the biodistributon of radioiodine in
tumor bearing nude mice at various time points after in-
jection of ^{125}I-benzoate-$(Z_{HER2 A})_2$. Data are combined from
10 two biodistribution experiments. Data for 4 h pi are av-
erages from both experiments.

Figure 14 shows the biodistributon of radioiodine in
tumor bearing nude mice 8 h after injection of ^{125}I-
benzoate-$(Z_{HER2 A})_2$.

15 Figure 15 shows a comparison of radioactivity con-
centration in blood and tumors. Data are combined from
two biodistribution experiments. A: experimental data. B:
curves fitted using non-linear regression with a two-
phase exponential decay model.

20 Figure 16 shows the tumor to blood ratio of radioac-
tivity concentration. Data are combined from two biodis-
tribution experiments.

Figure 17 is a whole body γ-camera image of tumor
bearing mice (SKOV-3) 6 h (left mouse) and 8 h (right
25 mouse) after iv tail injection of ^{125}I-benzoate-$(Z_{HER2 A})_2$
conjugate.

Figure 18 shows the biodistribution of radioactivity
in tumor bearing nude mice 4 hours after injection of
^{125}I-benzoate-$Z_{HER2 A}$.

30 Figure 19 shows the biodistribution of radioactivity
in tumor bearing nude mice 24 hours after injection of
^{125}I-benzoate-$Z_{HER2 A}$.

Figure 20 shows the kinetics of radioiodine in tumor
and blood of tumor bearing nude mice at various time
35 points after injection of ^{125}I-benzoate-$Z_{HER2 A}$.

Figure 21 shows the tumor to blood ratio of radioac-
tivity following injection of ^{125}I-benzoate-$Z_{HER2 A}$.

Figure 22 is a schematic illustration of the amino acid sequence of a fusion polypeptide produced in Example 6. $Z_{HER2\ A}$ represents HER2 binding domain with the sequence shown in SEQ ID NO:2 and ABD represents an albumin bind-

5   ing domain from streptococcal protein G.

Figure 23 shows the biodistribution of radioactivity in tumor bearing nude mice 12 (grey) and 24 hours (white) after injection of ^{125}I-benzoate-ABD$(Z_{HER2\ A})_2$.

Figure 24 shows the kinetics of radioiodine in tu-

10   mors of tumor bearing nude mice at various time points after injection of (A): ^{125}I-ABD$(Z_{HER2\ A})_2$, (B): ^{125}I-$(Z_{HER2\ A})_2$, and (C): ^{125}I-$Z_{HER2\ A}$.

Figure 25 shows a comparison of the delivered dose of the ^{125}I-benzoate-$Z_{HER2\ A}$, ^{125}I-benzoate-$(Z_{HER2\ A})_2$, and

15   ^{99m}Tc-$(Z_{HER2\ A})_2$.

Figure 26 shows the biodistribution of radioactivity in tumor bearing nude mice 8 hours after injection of ^{99m}Tc-$(Z_{HER2\ A})_2$.

Figure 27 shows a comparison of radioactivity in se-

20   lected organs in tumor bearing nude mice 8 hours after injection of ^{99m}Tc-$(Z_{HER2\ A})_2$.

Figure 28 is a diagram showing growth of SKBR-3 cells after exposure of ^{211}At-$(Z_{HER2\ A})_2$ for 24 hours. The filled, black circles of curve A represent cells without

25   exposure; the open squares of curve B represent cells exposed to a moderate level; and the filled grey squares of curve D represent cells exposed to high level of ^{211}At-$(Z_{HER2\ A})_2$. The filled grey triangles of curve C represent cells exposed to the high level of ^{211}At-$(Z_{HER2})_2$ combined

30   with a 500 fold excess of unlabeled His_6-$(Z_{HER2\ A})_2$. Values are averages of three experiments, error bars represent standard deviation.

Figure 29A and B show results from ABAS ELISA to determine the binding activity of clones picked from the

35   fourth and fifth round of the affinity maturation selection.

Figure 30 is a schematic illustration of the amino
acid sequence of the fusion polypeptide produced in Exam-
ples 9 and 10. Z_{HER2} represents a HER2 binding polypeptide
according to the first aspect of the invention, and His_6
5   represents a hexahistidyl tag.

Figure 31 is an overlay plot of sensograms obtained
from 10 affinity matured HER2 binding polypeptides ac-
cording to the invention, when injected over a HER2-
coated surface at an approximate concentration of 50 nM.
10   A: $Z_{HER2:205}$; B: $Z_{HER2:149}$; C: $Z_{HER2:202}$; D: $Z_{HER2\ A}$; E: $Z_{HER2:222}$;
F: $Z_{HER2:225}$; G: $Z_{HER2:209}$; H: $Z_{HER2:229}$; I: $Z_{HER2:207}$; J: $Z_{HER2:107}$;
K: $Z_{HER2:101}$. Note that $Z_{HER2\ A}$, isolated from the selection
with the first generation library, was added in the
study.

15   Figure 32 shows binding analysis results of Z_{HER2}
variants, which possess specific HER2 binding activity.
Resulting overlay sensograms from injections of (A)
$Z_{HER2:3053}$ (B) $Z_{HER2:0434}$ (C) $Z_{HER2:0024*}$ over sensor chip sur-
faces containing either HER2 or HIV-1 gp120 as indicated.
20   The invention will now be illustrated further
through the non-limiting recital of experiments conducted
in accordance therewith.

Example 1
25   ### Selection and study of HER2 binding polypeptides

In these experiments, several HER2 binding polypep-
tides according to the invention were selected from a li-
brary of a multitude of different SPA domain related
30   polypeptides, and subsequently characterized.

Library panning and clone selection
A combinatorial phage display library was prepared
essentially as described in Nord K et al (1995, *supra*).
35   The pool of this library which was used in the present
study comprised 8.7×10^8 variants of protein Z (Affi-
body® molecules), with random amino acid residues at po-

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sitions 9, 10, 11, 13, 14, 17, 18, 24, 25, 27, 28, 32 and
35. Antigen binding Affibody® molecules were selected in
four panning cycles using biotinylated human HER2 ex-
tracellular domain (HER2-ECD) as the target (recombinant
5   human HER2 extracellular domain, amino acids 238-2109,
provided by Fox Chase Cancer Center, Philadelphia, USA).
From the outcome of the four selection cycles, 91 clones
were picked for phage ELISA in order to perform an analy-
sis of their HER2 binding activity.
10

Phage ELISA for analysis of HER2 binding
        Phages from the clones obtained after four rounds of
selection were produced in 96 well plates, and an Enzyme
Linked ImmunoSorbent Assay (ELISA) was used for screening
15   for phages expressing HER2 binding Affibody® molecules.
Single colonies were used to inoculate 250 μl TSB medium
(30.0 g Tryptic Soy Broth (Merck), water to a final vol-
ume of 1 l, autoclaved) supplemented with 2 % glucose and
100 μg/ml ampicillin in a deep well 96 well plate and
20   grown on a shaker over night at 37 °C. 5 μl overnight
culture was added to 500 μl TSB+YE medium (30.0 g Tryptic
Soy Broth (Merck), 5.0 g yeast extract, water to a final
volume of 1 l, autoclaved) supplemented with 0.1 % glu-
cose and 100 μg/ml ampicillin in a new plate. After grow-
25   ing at 37 °C for 3 h, 0.5 μl of 5 x 10^{12} pfu/ml (2.5 x 10^9
pfu) helper phage M13K07 (New England Biolabs, #N0315S)
and 100 μl TSB+YE medium were added to each well, and the
plates were incubated without shaking at 37 °C for 30
minutes. 300 μl TSB+YE supplemented with IPTG, kanamycin
30   and ampicillin were added to each well to a final concen-
tration of 1 mM IPTG, 25 μg/ml kanamycin and 100 μg/ml
ampicillin, and the plates were incubated on a shaker
overnight at 30 °C. Cells were pelleted by centrifugation
at 2500 g for 15 minutes and supernatants, containing
35   phages expressing Affibody® molecules, were used in
ELISA. 100 μl of 4 μg/ml of HER2 in PBS (2.68 mM KCl, 137
mM NaCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) were

added to a microtiter plate (Nunc #446612) and incubated
for 1 month at 4 °C. After blocking wells with 2 % skim
milk powder in PBS (blocking buffer) for 1 h at room tem-
perature, 200 μl phage-containing supernatant and 50 μl

5   10 % blocking buffer were added. The plates were incu-
bated for 2 h at room temperature. A polyclonal antibody
(rabbit anti-M13, Abcam #ab6188) was diluted 1:1000 in
2 % blocking buffer, and 150 μl were added to each well.
The plate was incubated at room temperature for 1 h. A

10   goat anti-rabbit IgG antibody conjugated with alkaline
phosphatase (Sigma #A-3687) was diluted 1:10000 in 2 %
blocking buffer, after which 150 μl were added to each
well and incubated for 1 h at room temperature. Develop-
ing solution was prepared by dissolving Sigma-104 sub-

15   strate (Sigma #104-105) in a 1:1 mixture of 1 M dietha-
nolamine, 5 mM $MgCl_2$, pH 9.8 and water (1 tablet/5 ml of
1:1 mixture). Thereafter, 180 μl of the developing solu-
tion were added to each well. Wells were washed twice
with PBS-T (PBS + 0.1 % Tween-20) and once with PBS be-

20   fore addition of each new reagent. 25 minutes after addi-
tion of substrate, the plates were read at A_{405} in an
ELISA spectrophotometer (Basic Sunrise, Tecan).

Phages encoding HER2 binders were identified using a
threshold criterion of an ELISA value of A_{405} above 0.5.

25   48 clones gave an ELISA signal above this value, and were
selected for DNA sequence analysis, together with 5
clones selected at random for which no ELISA results were
available.

30   *DNA sequence analysis*

Sequencing of the DNA from the clones isolated ac-
cording to the procedure above was performed with the ABI
PRISM®, BigDye™ Terminator v2.0 Ready Reaction Cycle Se-
quencing Kit (Applied Biosystems) according to the manu-

35   facturer's recommendations. Plasmids were prepared and
DNA encoding the Affibody® molecules was sequenced using
the oligonucleotides RIT-27 (5'-GCTTCCGGCTCGTATGTTGTGTG-

3') and the biotinylated NOKA-2 (5'-biotin-CGGAACCAGAGCC-ACCACCGG-3'). The sequences were analyzed on an ABI PRISM® 3700 Genetic Analyser (Applied Biosystems). From the 53 clones previously selected, several clones were
5   found to encode the same amino acid sequence. Taking these degeneracies into account, four sequences of Affi-body® molecules expressed by clones selected in the ELISA binding assay are given in Figure 1 ($Z_{HER2\ A-D}$), and identi-fied in the sequence listing as SEQ ID NO:2-5.
10

Cloning and protein production
        Z_{HER2} polypeptides were expressed in *E. coli* cells, using expression vectors encoding constructs that are schematically illustrated in Figure 2. The polypeptides
15 were thereby produced as fusions to an N-terminal hexa-histidyl tag. The fusion polypeptides His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$ were purified on Immobilized Metal ion Affinity Chromatography (IMAC) columns and analyzed on SDS-PAGE. The result of the SDS-PAGE experiment is given in Figure
20 3.

Biosensor analysis of fusion polypeptides
        The interactions between the His-tagged Z_{HER2} vari-ants produced according to the preceding section and HER2
25 were analyzed using surface plasmon resonance in a Biacore® 2000 system (Biacore AB, Uppsala, Sweden). Human HER2, HIV-1 gp120 (Protein Sciences Corporation, #2003-MN), and BB (albumin-binding protein derived from strep-tococcal protein G), the latter two for use as controls,
30 were immobilized in different flow cells by amine cou-pling onto the carboxylated dextran layer on surfaces of CM-5 chips, according to the manufacturer's recommenda-tions. Immobilization of human HER2, HIV-1 gp120, and BB resulted in 1900, 6290, and 1000 resonance units (RU),
35 respectively. A fourth flow cell surface was activated and deactivated for use as blank during injections. The His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$ proteins were diluted in HBS (5

mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005 % surfactant P-
20, pH 7.4) to a final concentration of 10 μM, and in-
jected in random order as duplicates at a constant flow-
rate of 30 μl/minute. The ability of the purified pro-
teins His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$ to interact with HER2 was
confirmed, as illustrated by the sensorgrams of Figures 4
and 5, respectively.

Furthermore, kinetic studies were performed for
His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$. The CM-5 chip having 1900 RU of
human HER2 immobilized thereto was used. A series of six
different concentrations (1 μM - 40 μM) of HER2 binding
polypeptide was prepared in HBS for each of His_6-$Z_{HER2\ A}$
and His_6-$Z_{HER2\ B}$, and injected in random order as dupli-
cates at a flow-rate of 30 μl/minute. The total injection
time was 50 seconds (association) followed by a wash dur-
ing 6 minutes (dissociation). The surfaces were regener-
ated with 20 mM HCl for 10 seconds. The responses meas-
ured in reference cells (activated/deactivated surface)
were subtracted from the response measured in the cells
with immobilized HER2. The binding curves (sensorgrams)
were analyzed using the 1:1 Langmuir binding model of the
BIAevaluation 3.0.2 software (Biacore AB). As is clear
from the binding curves presented in Figures 6 (His_6-
$Z_{HER2\ A}$) and 7 (His_6-$Z_{HER2\ B}$), His_6-$Z_{HER2\ A}$ and His_6-$Z_{HER2\ B}$ both
clearly bind to HER2, as evidenced by the association and
dissociation curves with an indicated K_D of 10-100 nM for
His_6-$Z_{HER2\ A}$ and 200-400 nM for His_6-$Z_{HER2\ B}$. Furthermore, the
binding is selective, since neither of the HER2 binding
polypeptides studied bind to the BB and gp120 control an-
tigens (Figures 4 and 5).

In a second kinetic experiment, the His_6-$Z_{HER2\ A}$ and
His_6-$Z_{HER2\ B}$ variants were again injected over the HER2
surface at different concentrations (0-5 μM, with 0.0098
μM as the lowest concentration for His_6-$Z_{HER2\ A}$ and 0.0196
μM for His_6-$Z_{HER2\ B}$, diluted in HBS) with a flow rate of 30
μl/min. Prior to the kinetic analysis, the protein con-
centration had been determined by amino acid analysis.

32

The dissociation equilibrium constant (K_D), the associa-
tion rate constant (k_a), and the dissociation rate con-
stant (k_d) were calculated using BIAevaluation 3.2 soft-
ware (Biacore), assuming a one-to-one binding. For the
5   first two Biacore analyses, the samples were run at 25 °C
in duplicates in random order, and after each injection
the flow cells were regenerated by the injection of 10 mM
HCl. Upon evaluation of the binding curves (Figure 8A-
8B), the dissociation equilibrium constant (K_D) was de-
10 termined to be about 50 nM for His_6-$Z_{HER2\ A}$ and about 140
nM for His_6-$Z_{HER2\ B}$. The reason for the difference in K_D is
most likely due to the marked difference in dissociation
rate, as can be seen by comparing Figure 4, diagram A
with Figure 5, diagram A. For His_6-$Z_{HER2\ A}$, the association
15 rate constant (k_a) was calculated to be about 1.8×10^5
$M^{-1}s^{-1}$ and the dissociation rate constant (k_d) about
$9.9 \times 10^{-3}\ s^{-1}$, while for His_6-$Z_{HER2\ B}$, k_a and k_d were diffi-
cult to estimate due to the fast association and disso-
ciation kinetics. Thus, the His_6-$Z_{HER2\ A}$ affibody variant,
20 showing stronger binding to its target, was selected for
further characterization.

Example 2
Binding of $Z_{HER2\ A}$ to cells expressing HER2

25

Cell culture

    The human breast cancer cell line SKBR-3, known to
express about 2×10^6 HER2 molecules per cell, was pur-
chased from ATCC (ATCC #HTB-30). The cells were cultured
30 in RPMI 1640 medium supplemented with 10% fetal bovine
serum, 2 mM L-glutamine, and PEST (100 IU/ml penicillin
and 100 μg/ml streptomycin), all from Biochrom KG (Ber-
lin, Germany). The cells were cultured at 37 °C in hu-
midified air containing 5 % CO_2, and seeded in 3 cm petri
35 dishes three days before the experiment.

Radiolabeling

        Labeling precursor, N-succinimidyl *p*-(trimethyl-
stannyl)benzoate (SPMB), was prepared according to Orlova
et al in Nucl Med Biol 27:827-835 (2000), and 5 μg of
5   SPMB was added to 5 MBq of ^{125}I in a 5 % solution of ace-
tic acid. To start the reaction, 40 μg of chloramine-T
(Sigma, St. Louis, MO) in aqueous solution was added. The
reaction mixture was agitated for 5 min, and 80 μg of so-
dium-meta-bisulphate (Aldrich, Steinheim, Germany) in
10 aqueous solution was added to stop the reaction. The ra-
diolabeled precursor was added to 40 μg of His_6-$Z_{HER2 A}$ or
His_6-$Z_{HER2 B}$ in 0.07 M borate buffer, pH 9.2. The coupling
reaction was performed at room temperature for 45 min
with continuous shaking. Labeled Z_{HER2} variants were sepa-
15 rated from low molecular weight products using a NAP™-5
size exclusion column (Amersham Biosciences) equilibrated
with PBS. The radiolabeled Z_{HER2} variants were then ana-
lyzed using Biacore technology to verify that the label-
ing procedure had not affected the binding affinity to-
20 wards HER2-ECD. Both Z_{HER2} variants showed retained affin-
ity (data not shown).

Cellular tests

        To each dish of about 100000 SKBR-3 cells, 14 ng of
25 labeled His_6-$Z_{HER2 A}$ or His_6-$Z_{HER2 B}$ in 1 ml complemented me-
dium was added. This amount corresponds to a theoretical
ligand:receptor ratio of 5:1. Three dishes without cells
were treated in the same way, in order to determine un-
specific binding not derived from cells. This value was
30 subtracted from all others. To analyze the specificity of
cell binding, three dishes were treated not only with la-
beled Z_{HER2} variants, but also with a 500-fold excess of
unlabeled Z_{HER2} variants. After three hours of incubation
at 37 °C, the radioactive medium was removed and the
35 dishes were washed rapidly three times with ice-cold se-
rum-free medium. Cells were trypsinated with 0.5 ml Tryp-
sin/EDTA solution (0.25 %/0.02 % in PBS; Biochrom KG,

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Berlin, Germany) for 15 min at 37 °C. The cells were then
resuspended in 1 ml complemented medium, and 0.5 ml of
the cell suspension was used for cell counting and the
remaining 1 ml was used for radioactivity measurement in
5   an automated γ counter.

As presented in Figure 9, His_6-$Z_{HER2\ A}$ showed specific
binding to SKBR-3 cells, known to express 2×10^6 HER2
receptors per cell ("non-blocked" bar). The binding of
radiolabeled His_6-$Z_{HER2\ A}$ could be totally blocked by the
10   addition of an excess of non-labeled His_6-$Z_{HER2\ A}$
("blocked" bar). However, the binding of His_6-$Z_{HER2\ B}$ to
SKBR-3 cells was below the detection limit (data not
shown), probably as a result of the faster dissociation
rate for this Z_{HER2} variant (cf above).

15

Example 3
Expression and characterization of dimers of HER2 binding
polypeptides

20

DNA construction and protein production
The selection of a novel affibody ligand, denoted
His_6-$Z_{HER2\ A}$ and having affinity for the HER2 receptor, was
described above. A dimeric Z_{HER2} variant was constructed
25   by subcloning the gene fragment encoding the $Z_{HER2\ A}$ poly-
peptide into the expression vector for His_6-$Z_{HER2\ A}$. The
introduced $Z_{HER2\ A}$ fragment was verified by DNA sequencing
on a DNA sequencer ABI Prism® 3700 Analyzer (Applied Bio-
systems, Foster City, CA). The *Escherichia coli* strain
30   RR1ΔM15 (Rüther, Nucleic Acids Res 10:5765-5772 (1982))
was used as bacterial host during the cloning procedure.
The resulting vector encodes, under the control of the T7
promoter (Studier *et al*, Methods Enzymol 185:60-89
(1990)), a dimeric Z_{HER2} variant, $(Z_{HER2\ A})_2$, fused to an N-
35   terminal hexahistidyl (His_6) tag, which allows purifica-
tion by immobilized metal ion affinity chromatography
(IMAC).

The dimeric Z_{HER2} variant was expressed as a His_6-tagged fusion protein in *E. coli* strain BL21(DE3), and recovered by IMAC purification on a Talon Metal Affinity Resin (8901-2, BD Biosciences, CA) column under denatur-

5 ing conditions, as described for the monomeric polypep-
tides in Example 1. Renaturation of the purified His_6-$(Z_{HER2 A})_2$ fusion protein was performed by gel filtration using PD-10 columns, equilibrated with PBS (10 mM phos-phate, 154 mM NaCl, pH 7.1), according to the manufac-

10 turer's protocol (Amersham Biosciences). Protein concen-
tration was calculated from absorbance measurements at 280 nm, using the appropriate extinction coefficient (30440 $M^{-1}cm^{-1}$), and also verified by amino acid analysis (Aminosyraanalyscentralen, Uppsala, Sweden). The purified

15 protein was further analyzed by SDS-PAGE on a Tris-
Glycine 16 % homogenous gel, using a Novex system (Novex, CA, USA). Protein bands were visualized with Coomassie Brilliant Blue staining. Upon SDS-PAGE analysis, the pro-tein was observed as a specific band of the expected mo-

20 lecular weight (15.6 kD) (Figure 10, Lane 2 of insert).
Estimations from absorbance measurements at 280 nm demon-strated an expression level of about 200 mg/l of cell culture.

25 *Biosensor analysis*

A Biacore® 2000 instrument (Biacore AB) was used for real-time biospecific interaction analysis (BIA). A re-combinant extracellular domain of HER2 (HER2-ECD), di-luted in 10 mM NaAc, pH 4.5, was immobilized (about 2200

30 RU) on the carboxylated dextran layer of one flow-cell
surface of a CM5 sensor chip (research grade) (BR-1000-14, Biacore AB) by amine coupling according to the manufacturer's instructions. Another flow-cell surface was activated and deactivated, to serve as a reference

35 surface. For the Z_{HER2} sample, the buffer was changed to
HBS (5 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005 % surfactant P20, pH 7.4) by gel filtration using a NAP™-10 column, according to the manufacturer's protocol

according to the manufacturer's protocol (Amersham Bio-
sciences), and the sample was thereafter filtrated (0.45
μm; Millipore, Billerica, MA). Binding analyses were per-
formed at 25 °C, and HBS was used as running buffer. For
5   all Biacore analyses, the samples were run in duplicates
in random order, and after each injection the flow cells
were regenerated by the injection of 10 mM HCl.

In a first experiment, difference in binding to
HER2-ECD between the monomeric and the dimeric Z_{HER2} pro-
10   teins (His$_6$-$Z_{HER2\,A}$ of Example 1 and His$_6$-($Z_{HER2\,A}$)$_2$) was
tested by injection of 5 μM of each protein over the
HER2-ECD surface, with a flow rate of 5 μl/min. As can be
seen in Figure 10, a slower off-rate was observed for
His$_6$-($Z_{HER2\,A}$)$_2$, indicating a stronger binding between HER-
15   ECD and His$_6$-($Z_{HER2\,A}$)$_2$, compared to His$_6$-$Z_{HER2\,A}$.

In a second experiment, His$_6$-($Z_{HER2\,A}$)$_2$ was subjected
to a kinetic analysis, in which the protein was injected
over the HER2-ECD surface at different concentrations (0-
5 μM, with 0.0049 μM as the lowest concentration, diluted
20   in HBS) with a flow rate of 30 μl/min. Prior to the ki-
netic analysis, the protein concentration had been deter-
mined by amino acid analysis. The dissociation equilib-
rium constant (K_D), the association rate constant (k_a),
and the dissociation rate constant (k_d) were calculated
25   using BIAevaluation 3.2 software (Biacore AB), assuming
1:1 binding. Upon evaluation of the binding curves, the
dissociation equilibrium constant (K_D) was determined to
be about 3 nM, the association rate constant (k_a) was
calculated to be about 2.5 x 10^5 M^{-1} s^{-1} and the dissocia-
30   tion rate constant (k_d) about 7.6 x 10^{-4} s^{-1}. These values
can be compared to the kinetic constants obtained for the
monomeric His$_6$-$Z_{HER2\,A}$ of Example 1, confirming the
stronger binding of the dimeric His$_6$-($Z_{HER2\,A}$)$_2$. Such im-
proved apparent higher affinity, due to avidity effects
35   for the dimeric constructs, have been demonstrated ear-
lier for other Affibody® molecules (Gunneriusson E et al,
Protein Eng 12:873-878 (1999)).

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Example 4
Biodistribution and tumor targeting with $(Z_{HER2\ A})_2$ in nude
mice bearing SKOV-3 xenografts

5   In the experiments making up this example, the His_6-
tagged dimeric $(Z_{HER2\ A})_2$ polypeptide according to Example
3 was radiolabeled with ^{125}I and injected into mice bear-
ing a grafted tumor characterized by HER2 overexpression.
Studies of the biodistribution of the polypeptide were
10   conducted, as well as imaging of the injected mice to
study localization of the labeled polypeptide. A labeled
Z domain derivative with binding affinity for Taq DNA po-
lymerase was used as control without specificity for HER2
(Z_{Taq}; described in Gunneriusson E et al, supra and re-
15   ferred to therein as $Z_{Taq\ S1-1}$).

Materials and methods

Indirect radioiodination of $(Z_{HER2\ A})_2$
20   A volume of 2.3 μl ^{125}I (corresponding to 10 MBq)
($Na[^{125}I]$, Amersham Biosciences, Uppsala, Sweden) was
added to a siliconized microcentrifuge tube. 10 μl acetic
acid (0.1 % in water), 5 μl N-succinimidyl p-trimethyl-
stannyl-benzoate (1 mg/ml in 5 % acetic acid in methanol)
25   (prepared according to Koziorowski J et al, Appl Radiat
Isot 49:955-959 (1998)) and 10 μl Chloramine-T (4 mg/ml
in water) ($CH_3C_6H_4SO_2N(Cl)Na \cdot 3H_2O$, Sigma, St Louis, MO,
USA) was added. The reaction was allowed to take place
for five minutes with some mixing. The reaction was then
30   terminated with 10 μl sodium metabisulfate (8 mg/ml in
water) ($Na_2S_2O_5$, Sigma, St Louis, MO, USA). A volume of 40
μl $(Z_{HER2\ A})_2$ dimer (0.25 mg/ml in 0.07 M borate buffer, pH
9.2 (sodium borate, $Na_2B_4H_7 \cdot 10H_2O$, Sigma, St Louis, MO,
USA, and hydrochloric acid, HCl, Merck, Darmstadt, Ger-
35   many)) was added to the reaction tube. Another 40 μl of
borate buffer was added to each tube in order to raise
the pH to about 9. After a reaction time of 45 minutes

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with continuous shaking, the reaction components were
separated on NAP-5 size exclusion columns (Amersham Bio-
sciences, Uppsala, Sweden) equilibrated with PBS accord-
ing to the manufacturer's protocol. The reaction tube,
5   void fraction, high MW fraction, low MW fraction and col-
umn were measured at 60 cm (^{125}I) with a handheld γ detec-
tor (Mini-instruments Ltd, Essex, UK) in order to calcu-
late the labeling yield. The high molecular weight frac-
tion was stored in siliconized microcentrifuge tube at –
10   20 °C until use the following day. The obtained yield was
25-30 %.

Indirect radioiodination of Z_{Taq}

A stock solution of [^{125}I]-NaI was mixed with 10 μl
15   0.1 % aqueous solution of acetic acid, 5 μl of *N*-
succinimidyl *p*-trimethylstannyl-benzoate solution (1
mg/ml in 5% acetic acid in methanol), and 10 μl aqueous
solution of Chloramine-T (4 mg/ml). The reaction mixture
was vigorously vortexed, and incubated during 5 min at
20   room temperature with shaking. The reaction was quenched
with 10 μl aqueous solution of sodium metabisulfite (8
mg/ml). 21 μl solution of Z_{Taq} in PBS (2.4 mg/ml) was
added to the crude reaction mixture. The pH of the reac-
tion mixture pH was adjusted to about 9 by addition of
25   borate buffer (0.1 M, pH 9.15). The reaction mixture was
incubated at room temperature during 30 min with shaking,
and separated into high molecular weight fraction (la-
beled Z_{Taq}) and low molecular weight fraction on a NAP-5
column pre-equilibrated with 5 % albumin (bovine, frac-
30   tion V, Sigma, St. Louis, MO, USA) in PBS, using PBS as
eluent. The obtained radiochemical yield was between 75 %
and 80 %. The specific radioactivity was 100 kBq/μg.

Animal preparation

35   Female outbred nu/nu balb mice from M&B (10-12 weeks
old when arrived) were used under permission C181/1. Mice
were acclimatized in the animal facilities of the Rudbeck

laboratory, Uppsala, Sweden, using standard diet, bedding and environment during the week before xenografts were established in them. The mice had free access to food and drinking water.

5   Two months before the first experiment, 5×10^6 SKOV-3 human ovarian cancer cells (ATCC #HTB-77) were in-jected subcutaneously in the right hind leg of 33 mice. This group is denoted "Set A".

Three weeks before the second experiment, 10^7 SKOV-3
10   cells were injected subcutaneously in both hind legs of 32 mice. This group is denoted "Set B".

For imaging studies, two mice with big tumors (see below) were taken from Set A and all others from Set B.

By the time of the experiments, tumors had been es-
15   tablished in all mice, but were rather small and differed in size and status (encapsulated and invasive, stage of vascularization). At the time of use, all mice weighed 22-27 g.

20   *Biodistribution Experiment I*

20 mice from Set A were randomly divided into 5 groups (I-V) with 4 mice in each group. 2 mice from Set A with bigger tumors were excluded for use in imaging stud-ies. Groups, injections and times of sacrifice were
25   according to Scheme 1.

The mice were injected iv in the tail with 0.5 μg ($Z_{HER2\ A}$)$_2$, indirectly labeled with ^{125}I (100 kBq per mouse) in 50 μl PBS. Mice in group II ("blocked group") were pre-injected sc with 0.05 mg of unlabeled ($Z_{HER2\ A}$)$_2$ in 200
30   μl PBS, 45 min before the injection of labeled ($Z_{HER2\ A}$)$_2$. All injections were tolerated well, judging by the lack of any visible problems.

40

Scheme 1

Group	Mouse ID	Additional treatment	Time of sacrifice post injection (h)
I	1-4		1
II	5-8	Unlabeled $(Z_{HER2\ A})_2$	1
III	9-12		4
IV	13-16		8
V	17-20		24

5 min before the time of sacrifice, mice were in-
jected ip with a lethal dose of Ketalar/Rompun solution
5   (20 μl/g body weight, Ketalar 10 mg/ml (Pfizer, New York,
USA), Rompun 1 mg/ml (Bayer, Leverkusen, Germany)). Blood
was taken at the time of sacrifice by heart puncture with
a 1 ml syringe washed with diluted heparin (5000 IE/ml,
Leo Pharma, Copenhagen, Denmark). Blood, samples of
10   urine, muscle, bone, large and small intestines, heart,
bladder, lung, liver, spleen, pancreas, kidney, stomach,
salivary and thyroid glands, brain, tumors and tails were
dissected and collected in weighed 20 ml plastic bottles.
In the case of multiple tumors in some mice from Set B,
15   every tumor was collected in a separate bottle. The sam-
ples of organs and tissue were weighed, and their radio-
activity measured with a γ-counter (Automated γ-counter
with a 3-inch NaI(Tl) detector, 1480 Wallac WIZARD, Wal-
lac OY, Turku, Finland).
20

Biodistribution Experiment II
24 mice from Set B were randomly divided into 6
groups with 4 mice in each group. 8 mice from Set B with
bigger tumors were selected for use in imaging studies.
25   Groups, injections and times of sacrifice were according
to Scheme 2.
The mice of groups I and IV-VI were injected iv in
the tail with 0.5 μg $(Z_{HER2\ A})_2$, indirectly labeled with
^{125}I (100 kBq per mouse) in 50 μl PBS. The mice of group

II were injected with the same amount of radioiodinated
($Z_{HER2 A}$)$_2$, but subcutaneously in the tail. The mice of
group III ("negative control group") were injected iv in
the tail with 1.07 μg Z_{Taq} indirectly labeled with ^{125}I
5   (100 kBq per mouse) in 50 μl PBS. All injections were
tolerated well, judging by the lack of any visible prob-
lems.

Scheme 2

Group	Mouse ID	Additional treatment	Time of sacrifice post injection (h)
I	1-4		4
II	5-8		4
III	9-12	Labeled Z_{Taq}	4
IV	13-16		6
V	17-20		10
VI	21-24		15

10   Sacrifice and taking of samples were performed as
described above for Biodistribution Experiment I. In this
second experiment, the carcass was also collected and its
radioactivity content measured. The samples of organs and
tissue were weighed, and their radioactivity measured
15   with a γ-counter.

Measurement of radioactivity

A standard protocol for measurement of ^{125}I was used.
Counts per minute corrected with background level were
20   used for the evaluation. The tissue uptake value, ex-
pressed as %ID/g, percent injected dose per gram tissue,
was calculated as

$$\%ID/g = \frac{tissue\,radioactivity\,/\,injected\,radioactivity}{tissue\,weight} \times 100$$

25

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wherein for iv injections:

Injected radioactivity = Average radioactivity in control
                         syringes - Radioactivity in used
                         syringe - Radioactivity in tail

and for sc injections:

Injected radioactivity = Average radioactivity in control
                         syringes - Radioactivity in used
                         syringe

Imaging study

For the imaging, mice were divided in two groups with 5 mice in each, taking into account that every group would comprise one mouse with a big tumor from Set A. Mice from Set B were randomized. The two groups of mice were injected with 2.3 μg $(Z_{HER2\ A})_2$ indirectly labeled with ^{125}I (2.9 MBq per mouse) in 90 μl PBS 6 h or 8 h before imaging, respectively. All injections were tolerated well, judging by the lack of any visible problems.

Whole body imaging of the mice was performed at 6 and 8 h post injection (pi) of radioconjugate. Mice were forced to urinate, anesthetized with lethal Keta-lar/Rompun injection ip and killed by cervical disloca-tion. Mice (5 in each group) were placed in an e.CAM γ-camera (Siemens, Germany), and 10 min images were ob-tained at each time point. Two mice with bigger tumors (1 from each group) were selected for a special image in the same camera, with an exposure of 20 min. The images were acquired in a 256 x 256-bit matrix with a low energy, high resolution collimator in a 35 keV energy window with 99 % window size. The images were evaluated with the aid of Hermes software from Nuclear Diagnostics (Kent, UK).

Results

Blocking experiment

     The blocking experiment in Biodistribution Experi-
ment I was performed in order to establish whether uptake
of $(Z_{HER2\ A})_2$ in tumors was specific and receptor regu-
lated. Before the major iv injection of radioiodinated
dimer, 0.05 mg of unlabeled $(Z_{HER2\ A})_2$ was injected sc in
the mice of group II of Scheme 1. Uptake of radioactivity
at 1 h post injection in group I and group II was com-
pared. Tumor to blood ratios for the two groups of mice
were 0.72 (group I, average) and 0.25 (group II, average)
(Figure 11). However, the difference in uptake was not
significant (p = 0.16). In all organs except the tumor,
radioactivity uptake was the same for both blocked and
non-blocked animals.

     The rather low tumor to blood ratio in the case of
non-blocked mice can be explained by the early time point
(1 h pi) chosen for this experiment.

Specificity of uptake

     In Biodistribution Experiment II, the mice of group
III were injected with an amount of Z_{Taq} corresponding to
the amount of $(Z_{HER2\ A})_2$ injected in the mice of the other
groups. Z_{Taq} had been labeled with radioiodine using the
same indirect method as for $(Z_{HER2\ A})_2$. Z_{Taq} is non-specific
with respect to HER2 receptors. Uptake of radioactivity
at 4 h post injection in group I and group III were com-
pared. The results of this experiment (Figure 12) showed
that the non-specific Z_{Taq} molecule had a lower tumor up-
take than the specific $(Z_{HER2\ A})_2$ molecule. Tumor to blood
ratios in this experiment were 1.43 (group I, specific
$(Z_{HER2\ A})_2$) and 0.15 (group III, unspecific Z_{Taq}). Statisti-
cal analysis showed that the difference was significant
(p = 0.009). In all other organs, radioactivity uptake
was on the same level for both Z molecules.

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A higher tumor to blood ratio for $(Z_{HER2\ A})_2$ was observed in this experiment, compared to the blocking experiment. This was likely due to the later time point of the experiment (4 h pi).

Biodistribution

The results from the groups of mice in Biodistribution Experiments I and II that had been injected iv with labeled $(Z_{HER2\ A})_2$ were combined in an analysis of the biodistribution of radioiodine in organs and tissues of the tumor bearing mice. The results are shown in Figures 13-16.

Referring to Figure 13 and 14, the concentration of ^{125}I in tumors was higher than in most normal organs, indicating that the $(Z_{HER2\ A})_2$ polypeptide is able to target tumor cells bearing HER2. The radioactivity concentration in normal organs and tissues was found to be lower than in the tumors, with the exception of kidney (all time points), thyroid (all time points), and liver (early time points). The experiments showed a quick clearance of radioiodine from blood and normal organs. Clearance from normal organs mainly followed blood clearance, with the exception of the thyroid, where accumulation of radioiodine was found. Elevated thyroid uptake of free iodine, even for indirect labeling methods, is well known and can to some extent be prevented with "cold", or non-radioactive, iodine (Larsen RH *et al*, Nucl Med Biol 25:351-357 (1998)). High concentrations of radioiodine were also found in the kidneys of the mice, which was also expected since the kidneys are the main excretion pathway for such small proteins and catabolites.

Figures 15A and B show the progression over time of the radioiodine concentration in blood and in tumor. Starting at 4 h pi, tumor radioactivity was higher than blood radioactivity. Using the experimental data as presented in Figure 15A, the half-lives of radioiodine in blood and tumors were calculated using GraphPad Prism©, v

3.0, from GraphPad Software (San Diego, USA). Non-linear regression with a two-phase exponential decay was used as model, and the resulting graphs are presented in Figure 15B. $T_{\frac{1}{2}}\alpha$ for the tumors was shorter (0.36 h) than for
5   blood (0.76 h), but $T_{\frac{1}{2}}\beta$ was longer (87.5 h for tumors vs 4.3 h for blood), which is in good agreement with the obtained results. For comparison, $T_{\frac{1}{2}}\alpha$ in blood for the labeled dimer, calculated with the same model using biodistribution data from normal, non-tumor bearing mice, was
10 0.3 h (data not shown).

The tumor to blood ratio of radioactivity concentration (Figure 16) increased with time during at least 12 h pi. This ratio is a good estimating factor for imaging contrast, because the main background in radioactivity
15 imaging comes from radioactivity in the blood. Taking into account the tumor to blood ratio and the tumors' radioactivity concentrations, it was concluded that 6 and 8 h pi might be the optimal time points for images. For an image with good contrast, the tumor to non-tumor radioac-
20 tivity concentration ratio should not be less than 2.

Gamma images

Gamma images were taken of each of the two groups of 5 mice each selected for imaging (at 6 h and 8 h pi, re-
25 spectively). In all mice at both time points, kidneys with well-defined structures could be identified. Some additional structure (probably liver) is visible on the image of mice 6 h pi over the kidneys, as well as an elevated background from a generalized blood pool. Some ani-
30 mals had some urine in their bladders, which is also directly visible. On the image with mice 8 h pi, additional structure in the neck area can be identified, which in all likelihood is the thyroid.

From 5 animals in each image session, one large in-
35 vasive tumor (from the first batch of SKOV-3 injection) was visualized. No other tumor localization was evident.

The two animals in question were chosen for an additional imaging session, and the result is shown in Figure 17.

Summary

5   Biodistribution of the dimer polypeptide $(Z_{HER2\ A})_2$, indirectly labeled with radioiodine (^{125}I) via a benzoate group, in mice bearing SKOV-3 (ovarian cancer cell line) tumors showed good agreement with normal biodistribution of the conjugate in normal mice. Tumor uptake of radioio-

10   dine injected as ^{125}I-benzoate-$(Z_{HER2\ A})_2$ was achieved. The tumor uptake was receptor mediated and specific, as shown by blocking experiments using a pre-injection of a high concentration of non-labeled $(Z_{HER2\ A})_2$ molecule, and by injection of a non-specific, labeled Z variant, Z_{Taq}.

15   Analysis of the data obtained showed that the radioactivity concentration in tumor was higher than the radioactivity concentration in blood after 4 h pi, and higher than in the majority of normal organs and tissues after 6 h pi, except for the kidneys and thyroid. Gamma images of

20   mice bearing SKOV-3 xenograft tumors were obtained at 6 and 8 h pi. Good resolution was achieved. At both time points, big invasive tumors could be clearly identified.

25   Example 5
        Biodistribution and tumor targeting with His_6-$Z_{HER2\ A}$ mono-
               mer in nude mice bearing SKOV-3 xenografts

        In the experiments making up this example, the mono-

30   meric $Z_{HER2\ A}$ polypeptide according to Example 1 and 2 was radiolabeled with ^{125}I and injected into mice bearing a grafted tumor characterized by HER2 overexpression. Studies of the biodistribution of the polypeptide were conducted to study its localization.

35

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Materials and methods

Indirect radioiodination of $Z_{HER2\ A}$

5   For labeling with ^{125}I, 40 μl of monomeric His_6-$Z_{HER2\ A}$
was subjected to the same treatment as the dimeric poly-
peptide construct in Example 4.

Animal preparation

      Female outbred nu/nu balb mice from M&B (10-12 weeks
10   old when arrived) were used under permission C66/4. Mice
were acclimatized in the animal facilities of the Rudbeck
laboratory, Uppsala, Sweden, using standard diet, bedding
and environment during the week before xenografts were
established in them. The mice had free access to food and
15   drinking water. Three weeks before the experiment,
5 x 10^7 SKOV-3 human ovarian cancer cells (ATCC #HTB-77)
were injected subcutaneously in the right hind leg of 16
mice. By the time of the experiments, tumors had been es-
tablished in all mice and all mice weighed 22-27 g.
20

Measurement of radioactivity

      ^{125}I measurement was performed and %ID/g calculated
as described in Example 4.

25   *Biodistribution Experiment*

      16 mice were randomly divided into 4 groups (I-IV)
with 4 mice in each group. Groups, injections and times
of sacrifice were according to Scheme 3. The mice were
injected iv in the tail with 0.5 μg $Z_{HER2\ A}$, indirectly la-
30   beled with ^{125}I (100 kBq per mouse) in 50 μl PBS. All in-
jections were tolerated well, judging by the lack of any
visible problems.

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Scheme 3

Group	Mouse ID	Time of sacrifice post injection (h)
I	1-4	1
II	5-8	4
III	9-12	8
IV	13-16	24

The mice were sacrificed and samples taken as de-
5   scribed in the first biodistribution study of Example 4.
The samples of organs and tissue were weighed, and their
radioactivity measured with a γ-counter (Automated γ-
counter with a 3-inch NaI(Tl) detector, 1480 Wallac
WIZARD, Wallac OY, Turku, Finland).

10

Results

The results from the groups of mice that had been
injected sc with labeled $Z_{HER2\ A}$ were analyzed to establish
the distribution of radioiodine in organs and tissues of
15   the mice. The results are shown in Figures 18-20. Refer-
ring to Figure 18 and 19, the concentration of ^{125}I in tu-
mors was higher than in most normal organs at 4 and 24
hours, indicating that the $Z_{HER2\ A}$ polypeptide is able to
target tumor cells bearing HER2. The radioactivity con-
20   centration in normal organs and tissues was found to be
lower than in the tumors, with the exception of kidney
(all time points) and thyroid (all time points). The ex-
periments showed a quick clearance of radioiodine from
blood and normal organs. Clearance from normal organs
25   mainly followed blood clearance, with the exception of
the thyroid, where accumulation of radioiodine was found.
High concentrations of radioiodine were also found in the
kidneys of the mice. Figure 20 shows the progression over
time of the radioiodine concentration in blood and in tu-
30   mor. Starting at 4 h pi, tumor radioactivity was six
times higher than blood radioactivity. The tumor to blood
ratio of radioactivity concentration (Figure 21) in-

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creased with time during at least 8 h pi, when it was 10
to 1.

Summary

5   Biodistribution of the monomer polypeptide $Z_{HER2\ A}$,
indirectly labeled with radioiodine (^{125}I) via a benzoate
group, in mice bearing SKOV-3 (ovarian cancer cell line)
tumors showed good agreement with normal biodistribution
of the conjugate in normal mice. Tumor uptake of radioio-
10   dine injected as ^{125}I-benzoate-$Z_{HER2\ A}$ was achieved. Analy-
sis of the data obtained showed that the radioactivity
concentration in tumor was higher than the radioactivity
concentration in blood after 4 h pi, and in contrast to
the dimeric form $(Z_{HER2\ A})_2$, also higher than in the major-
15   ity of normal organs and tissues already after 4 h pi,
except for the kidneys and thyroid.

Example 6
20   Biodistribution and tumor targeting with $ABD(Z_{HER2\ A})_2$ in
nude mice bearing SKOV-3 xenografts

        In the experiments making up this example, a dimeric
$(Z_{HER2\ A})_2$ polypeptide according to Example 3 was geneti-
25   cally fused at its N-terminal end to "Albumin Binding Do-
main" (ABD) from streptococcal protein G, in order to
form a polypeptide denoted $ABD(Z_{HER2\ A})_2$ (Figure 22). ABD
binds human and mouse serum albumin with high affinity (M
Johansson et al, J Biol Chem 277:8114-8120 (2002)). Albu-
30   min is an abundant protein in the blood with a slow
plasma clearance. Binding to albumin with high affinity
should confer upon the binder slow kinetics in similar to
the albumin protein itself. Extending the circulation
time of a tumor targeting ligand in an animal should, in
35   theory, enhance the dose delivered to the tumor. To test
this, the $ABD(Z_{HER2\ A})_2$ polypeptide was radiolabeled with
^{125}I and injected into mice bearing a grafted tumor char-

50

acterized by HER2 overexpression. Studies of the biodis-
tribution of the polypeptide were conducted to study its
localization.

5   Materials and methods

DNA construction and protein production

The selection of a novel affibody ligand, denoted
His_6-$Z_{HER2\ A}$ and having affinity for the HER2 receptor, is

10 described above in Examples 1 and 2. A dimeric $Z_{HER2\ A}$
variant, constructed by subcloning the gene fragment en-
coding the $Z_{HER2\ A}$ polypeptide into the expression vector
for His_6-$Z_{HER2\ A}$, was described in Example 3. An ABD fusion
protein of this dimeric $Z_{HER2\ A}$ variant was constructed by

15 subcloning the gene fragment encoding the ABD polypeptide
into the expression vector for His_6-$Z_{HER2\ A}$, substituting
the hexahistidine tag with the ABD polypeptide. In addi-
tion, a C-terminal cysteine residue was added to the fu-
sion protein using extension PCR. The introduced ABD

20 fragment was verified by DNA sequencing on a DNA se-
quencer ABI Prism® 3700 Analyzer (Applied Biosystems,
Foster City, CA). The Escherichia coli strain RR1ΔM15
(Rüther, Nucleic Acids Res 10:5765-5772 (1982)) was used
as bacterial host during the cloning procedure. The re-

25 sulting vector encodes, under the control of the T7 pro-
moter (Studier et al, Meth Enzymol 185:60-89 (1990)), an
ABD fused dimeric Z_{HER2} variant, ABD($Z_{HER2\ A}$)$_2$, fused to a C-
terminal cysteine residue, which allows site specific
chemical modifications (Figure 22). The dimeric Z_{HER2} vari-

30 ant was expressed as a fusion with ABD in E. coli strain
BL21(DE3), and recovered by affinity chromatography puri-
fication on an albumin Sepharose affinity column prepared
according to the manufacturer's protocol (Amersham Bio-
sciences). Protein concentration was calculated from ab-

35 sorbance measurements at 280 nm, using the appropriate
extinction coefficient. The purified protein was further
analyzed by SDS-PAGE on a Tris-Glycine 16 % homogenous

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gel, using a Novex system (Novex, CA, USA). Protein bands
were visualized with Coomassie Brilliant Blue staining.
Upon SDS-PAGE analysis, the protein was observed as a
specific band of the expected molecular weight.

5

Indirect radioiodination of ABD($Z_{HER2\ A}$)$_2$

For labeling with ^{125}I, 40 μl of ABD($Z_{HER2\ A}$)$_2$ was sub-
jected to the same treatment as the dimeric polypeptide
construct in Example 4.

10

Animal preparation

Female outbred nu/nu balb mice from M&B (10-12 weeks
old when arrived) were used under permission C66/4. Mice
were acclimatized in the animal facilities of the Rudbeck
15   laboratory, Uppsala, Sweden, using standard diet, bedding
and environment during the week before xenografts were
established in them. The mice had free access to food and
drinking water. Three weeks before the experiment,
5 x 10^7 SKOV-3 human ovarian cancer cells (ATCC #HTB-77)
20   were injected subcutaneously into the right hind leg of
16 mice. By the time of the experiments, tumors had been
established in all mice and all mice weighed 22-27 g.

Measurement of radioactivity

25   ^{125}I measurement was performed and %ID/g calculated
as described in Example 4.

Biodistribution Experiment

16 mice were randomly divided into 4 groups (I-V)
30   with 4 mice in each group. Groups, injections and times
of sacrifice were according to Scheme 4. The mice were
injected iv in the tail with 0.5 μg ABD($Z_{HER2\ A}$)$_2$, indi-
rectly labeled with ^{125}I (100 kBq per mouse) in 50 μl PBS.
All injections were tolerated well, judging by the lack
35   of any visible problems.

Scheme 4

Group	Mouse ID	Time of sacrifice post injection (h)
I	1-4	12
II	5-8	24
III	9-12	48
IV	13-16	72

The mice were sacrificed and samples taken as de-
scribed in the first biodistribution study of Example 4.
5   The samples of organs and tissue were weighed, and their
radioactivity measured with a γ-counter (Automated γ-
counter with a 3-inch NaI(Tl) detector, 1480 Wallac
WIZARD, Wallac OY, Turku, Finland).

10 Results

The results are shown in Figures 23-24. Referring to
Figure 23, the concentration of ^{125}I in tumors was higher
than in most normal organs at 12 (tumor 2.4 %ID/g) and 24
hours (tumor 2.2 %ID/g), indicating that the ABD($Z_{HER2\ A}$)$_2$
15 polypeptide is able to target tumor cells bearing HER2.
The radioactivity concentration in normal organs and tis-
sues was found to be lower than in the tumors, with the
exception of kidney (all time points, at 24 hours 3.5
%ID/g), thyroid (all time points, at 24 hours 5.2 %ID/g)
20 and blood (all time points, at 24 hours 3.9 %ID/g). Lung
values are more or less equivalent to the tumor values,
which may be due to the high blood content in the lungs.
The experiments showed very slow clearance of radioiodine
from blood. This is to be expected, since the ABD moiety
25 of ABD($Z_{HER2\ A}$)$_2$ binds with high affinity to serum albumin,
an abundant protein in the blood with slow kinetics.
Clearance from normal organs was faster than blood clear-
ance, with the exception of the thyroid, where accumula-
tion of radioiodine was found. High concentrations of ra-
30 dioiodine were also found in the kidneys of the mice.
Figure 24 shows the progression over time of the radioio-
dine concentration associated with ABD($Z_{HER2\ A}$)$_2$ in tumor,

in comparison with the results generated for monomeric
and dimeric constructs of $Z_{HER2\ A}$ in Examples 4 and 5. At
24 h pi, tumor radioactivity using ABD$(Z_{HER2\ A})_2$ was thir-
teen times higher than for monomer or dimer, indicating

5   that extending residence time of the targeting moiety in
the body indeed may be used to increase the dose on the
tumor. The data also support that the $(Z_{HER2\ A})_2$ moiety can
be functionally coupled to the albumin binding domain.

10   Summary

         Biodistribution of the fusion polypeptide
ABD$(Z_{HER2\ A})_2$, indirectly labeled with radioiodine (^{125}I)
via a benzoate group, in mice bearing SKOV-3 (ovarian
cancer cell line) tumors showed good agreement with the

15   expected properties of an albumin binding polypeptide.
Tumor uptake of radioiodine injected as ^{125}I-ABD$(Z_{HER2\ A})_2$
was achieved, and the dose on the tumor was higher than
for the dimeric or monomeric versions of the $Z_{HER2\ A}$ poly-
peptide. Analysis of the data obtained showed that the

20   radioactivity concentration in tumor was higher than the
radioactivity concentration in most other organs after 12
h pi, although the radioactivity in lungs, kidneys, thy-
roid and blood remained high.

25

                            Example 7
Biodistribution of technetium-labeled dimer ^{99m}Tc-$(Z_{HER2\ A})_2$

         In the experiments making up this example, the

30   dimeric His$_6$-$(Z_{HER2\ A})_2$ polypeptide according to Example 3
was labeled with the diagnostic imaging nuclide 99m-
technetieum and injected into normal mice and into mice
bearing a grafted tumor characterized by HER2 overexpres-
sion. ^{99m}Technetieum (^{99m}Tc) is a radionuclide with close

35   to ideal properties for *in vivo* diagnosis. In addition,
it is cheap and readily available, making it a good can-
didate for medical imaging in the clinic. Studies of bio-

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distribution of the polypeptide were conducted to study its localization.

Materials and methods

5

Technetium labeling of His_6-$(Z_{HER2\ A})_2$

The His_6-$(Z_{HER2\ A})_2$ polypeptide was labeled according to manufacturer's instructions using the commercially available IsoLink™ kit (Mallinckrodt, Netherlands), which
10   directs the ^{99m}Tc nuclide to the hexahistidine tag on the His_6-$(Z_{HER2\ A})_2$ protein. The IsoLink™ kit reagents generate a technetium cation, $[^{99m}Tc(CO)_3(H_2O)_3]^+$, which can then be stably coordinated by the hexahistidine tag, providing means for labelling the His_6 tag of the His_6-$(Z_{HER2\ A})_2$
15   polypeptide specifically.

Briefly, 0.5-0.6 ml ^{99m}Tc stock solution eluted from the generator at Uppsala University Hospital was incubated with the content of the IsoLink™ Carbonyl labelling agent (DRN4335, Mallinckrodt) for 20 min at 100 °C in a
20   water bath. 40 μl of the thus obtained technetium cation, $[^{99m}Tc(CO)_3(H_2O)_3]^+$, was mixed with 40 μl His_6-$(Z_{HER2\ A})_2$ in PBS (1.2 mg/ml) and incubated for 1 h at 50 °C. The product was purified by separation on a NAP-5 size exclusion column with a cut-off of 5 kD (Pharmacia, Uppsala). The
25   product purity was 97 %, according to instant thin layer chromatography.

Animal preparation

Female outbred nu/nu balb mice from M&B (10-12 weeks
30   old when arrived) were used under permission C66/4. Mice were acclimatized in the animal facilities of the Rudbeck laboratory, Uppsala, Sweden, using standard diet, bedding and environment during the week before xenografts were established in them. The mice had free access to food and
35   drinking water. Three weeks before the experiment, 5 x 10^7 SKOV-3 human ovarian cancer cells (ATCC #HTB-77) were injected subcutaneously in the right hind leg of 4

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mice. By the time of the experiments, tumors had been es-
tablished in all mice and all mice weighed 22-27 g.

Measurement of radioactivity

5   ^{99m}Tc measurement was performed and %ID/g calculated
as described for ^{125}I in Example 4.

Biodistribution Experiment

        One group of mice with 4 animals was used for the
10 experiment. Injections and times of sacrifice were ac-
cording to Scheme 5. The mice were injected iv in the
tail with 0.5 μg His$_6$-(Z$_{HER2\ A}$)$_2$, labeled with ^{99m}Tc (100 kBq
per mouse) in 50 μl PBS. All injections were tolerated
well, judging by the lack of any visible problems.

15

Scheme 5

Group	Mouse ID	Time of sacrifice post injection (h)
I	1-4	8

        The mice were sacrificed and samples taken as de-
scribed in the first biodistribution study of Example 4.
20 The samples of organs and tissue were weighed, and their
radioactivity measured with a γ-counter (Automated γ-
counter with a 3-inch NaI(Tl) detector, 1480 Wallac
WIZARD, Wallac OY, Turku, Finland).

25 <u>Results</u>

        The conjugate ^{99m}Tc-His$_6$-(Z$_{HER2\ A}$)$_2$ had a high stability
in vitro (challenge with PBS, plasma, cysteine and excess
of free histidine). The results from the *in vivo* biodis-
tribution ^{99m}Tc-His$_6$-(Z$_{HER2\ A}$)$_2$ experiments are shown in Fig-
30 ures 25-27. Referring to Figure 25, the total tumor dose
was three times higher using ^{99m}Tc-His$_6$-(Z$_{HER2\ A}$)$_2$ (3.3 %ID/g
at 8 h pi) as compared to the concentration of the ^{125}I-
labeled monomeric construct, ^{125}I-benzoate-Z$_{HER2\ A}$ (1.1
%ID/g at 8 h pi), or dimeric construct, ^{125}I-benzoate-
35 (Z$_{HER2\ A}$)$_2$ (1.06 %ID/g at 8 h pi). It is known that ^{99m}Tc is

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a better residualizing agent than ^{125}I, implying that the
delivered dose remains, whereas iodine doses are metabo-
lized and released from the tumor cells. Referring to
Figure 26, the concentration of ^{99m}Tc in tumors was higher

5   than in most normal organs at 8 h pi (tumor 3.3 %ID/g),
indicating that the ^{99m}Tc-His$_6$-(Z$_{HER2\ A}$)$_2$ polypeptide is able
to target tumor cells bearing HER2. The radioactivity
concentration in normal organs and tissues was found to
be lower than in the tumors, with the exception of kidney

10   (at 8 hours 27 %ID/g, 36 x the tumor dose) and liver (at
8 hours 11.4 %ID/g, 3 x the tumor dose) (Figure 27). High
kidney values were expected, since ^{99m}Tc-His$_6$-(Z$_{HER2\ A}$)$_2$ is
cleared via the kidneys. The high level of tracer in the
kidneys does not present a toxicity problem, since the

15   total dose injected will be very low for diagnostic pur-
poses. The amount of radioactivity in thyroid was not at
all as high as when ^{125}I was used (Example 4), as expected
(at 8 hours 1 %ID/g). Clearance from normal organs mainly
followed blood clearance, with the exception of the kid-

20   neys, where accumulation of ^{99m}technetium was observed
with a peak at one hour. The levels thereafter decreased,
with a rapid elimination phase during one hour (up to 2
hours pi) followed by a slow elimination phase, with a
substantial dose (70 %ID/g) still in the kidney after 24

25   hours.

Tumor to blood ratio for the technetium conjugate at
8 hours pi was 4, with a total tumor dose of 3.3 %ID/g.
This indicated that ^{99m}technetium could be used for *in
vivo* diagnostic applications. For comparison, at the same

30   time point, the tumor doses of ^{125}I-benzoate-(Z$_{HER2\ A}$)$_2$ and
^{125}I-benzoate-Z$_{HER2\ A}$ were about 1 %, but the tumor to blood
ratios were about 11.

Summary

35   Biodistribution of the ^{99m}Tc labeled polypeptide
^{99m}Tc-His$_6$-(Z$_{HER2\ A}$)$_2$ in mice bearing SKOV-3 (ovarian cancer
cell line) tumors showed relatively good distribution

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properties for medical imaging purposes. Tumor uptake of technetium injected as ^{99m}Tc-His$_6$-(Z$_{HER2\ A}$)$_2$ was achieved and the dose on the tumor was higher than for ^{125}I-labeled dimeric or monomeric versions of the Z$_{HER2\ A}$ polypeptide.

5 Analysis of the data obtained showed that the radioactivity concentration in tumor was higher than the radioactivity concentration in most other organs and blood after 8 h pi, although the liver and the kidney had substantially higher uptake than the tumor.

10

Example 8
In vitro labelling and characterization using ^{211}At

15   In the experiments making up this example, the dimeric His$_6$-(Z$_{HER2\ A}$)$_2$ polypeptide according to Example 3 was labeled with the therapeutic radio nuclide ^{211}astatine (^{211}At), using the same chemistry as described earlier for labeling with ^{125}I. One goal for radionuclide tumor tar-

20 geting is radiation therapy. This requires nuclides that emit high energy particles, such as α particles and high energy β particles, which can eradicate the targeted cell. The α emitting radiohalogen ^{211}At ($T_{½}$ = 7.2 h) has a range of just a few cell diameters, which means that cell

25 eradication may take place with high precision. Studies of the cell killing ability *in vitro* of the ^{211}At-labeled polypeptide were conducted.

Materials and methods

30   Two days in advance, 100 000 SKBR-3 cells, characterized by HER2 overexpression, were seeded in 3 cm dishes. 60 μg His$_6$-(Z$_{HER2\ A}$)$_2$ polypeptide was labeled with ^{211}At produced in the Scanditronix MC32 cyclotron of the Copenhagen University Hospital and purified in the labo-

35 ratory of Jörgen Carlsson, Uppsala University, Uppsala, Sweden. An approximate 1:1 and 5:1 molar ratio of ^{211}At-(Z$_{HER2\ A}$)$_2$ molecules per cell receptor were added to three

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dishes each. Three additional dishes were supplied with
the 5:1 concentration of ^{211}At-$(Z_{HER2\ A})_2$ but also a 500
times access of unlabeled His_6-$(Z_{HER2\ A})_2$ to block the bind-
ing sites and thereby estimate effects of unspecific ir-
5   radiation. Cells were incubated with ^{211}At-$(Z_{HER2\ A})_2$ for 24
hours in 37 °C. After 24 hours of incubation, all cells
were washed and supplied with new, fresh medium. They
were then monitored for growth once a week for about two
months.

10   In parallel, a set of dishes prepared in the same
way as those used in the cell killing assay described
above was supplied with the same solutions of ^{211}At-
$(Z_{HER2\ A})_2$. These cells were harvested at different time
points, and cell numbers counted and radioactivity con-
15 tent measured, in order to establish uptake curves for
all groups of dishes. These curves were used to calculate
the amount of decays per cell over the 24 hour period
used in the cell killing experiment.

20 Results

        Cells from the human breast cancer cell line SKBR-3,
which overexpresses HER-2, were exposed to two different
doses of ^{211}At-$(Z_{HER2\ A})_2$ for 24 hours. In the first dose,
the added ^{211}At-$(Z_{HER2\ A})_2$ molecules equaled the number of
25 HER2 target receptors on the cells. In the second dose,
^{211}At-labeled molecules were added in a five-fold excess.
After 24 hours of incubation, the cells were monitored
and counted once a week for two months, and the surviving
fraction determined. As seen in Figure 28, the response
30 was related to the dose given. The cells receiving the
equimolar amount of ^{211}At-$(Z_{HER2\ A})_2$ did not show any spe-
cific growth delay as compared with the control where ra-
dioactivity was added without a targeting agent. There
was a short growth delay due to unspecific radiation dam-
35 age, but the cells did not stop growing. In contrast,
when ^{211}At-$(Z_{HER2\ A})_2$ was added in fivefold excess, the in-
hibition of cell growth was impressive. At the end of the

experiment, the group receiving the high dose had not yet
recovered, whereas the low dose and the control groups
had increased by 10^6. Assuming that surviving cells re-
tain about the same growth rate after irradiation as be-

5   fore, all cells in the 5:1 group were eradicated. The up-
take curve revealed that the 5:1 blocked group also had
received substantial cell associated irradiation, proba-
bly due to insufficient blockage. The number of decays
per cell (DPC) was estimated from integrated uptake

10   curves. From the growth curve, doubling times were calcu-
lated as the slope of the exponential growth curve, and
surviving fraction was calculated by extrapolation of de-
layed growth back to the time of exposure. Results are
shown in Table 1.

15

Table 1

	Doubling time (days)	DPC	Survival
Control	2.5	-	100 %
Block	2.6	12	7.0 %
1:1	2.5	37	3.4 %
5:1	-	98	-

Summary

     ^{211}At-$(Z_{HER2})_2$ showed specific binding to HER2 overex-
pressing SKBR-3 cells *in vitro*. Induced cell death corre-

20   lated well with the accumulated dose. Less than 100 de-
cays per cell were shown to be enough to cause killing of
single cells and to achieve complete cell eradication.

25   Example 9
     Identification and characterization of additional HER2-
                    binding polypeptides

     In order to increase the affinity of the HER2 bind-
30   ing Z variants obtained from the selection described in

Example 1, an affinity maturation strategy was applied involving the construction of a second library (Gunneriusson *et al*, Protein Eng 12:873-878 (1999); Nord *et al*, Eur J Biochem 268:4269-77 (2001)), followed by rese-
5   lection against HER2. An alignment of the first genera-
tion of polypeptide variants $Z_{HER2\ A,\ B,\ C\ and\ D}$ showed that, apart from the identities at position, 13, 14, 28, 32 and 35, between $Z_{HER2\ A}$ and $Z_{HER2\ B}$, there is a convergence of R and K in position 10 and of Q and T in position 11. Thus,
10 the second generation library contained five fixed posi-
tions 13, 14, 28, 32 and 35 and two partly fixed posi-
tions, position 10 (cgc/aaa as degenerated codons) and position 11 (caa/acc as degenerated codons), while the remaining positions 9, 17, 18, 24, 25 and 27 were again
15 randomized using NNG/T degenerated codons. After trans-
formation, a library of 3 x 10^8 clones was obtained. An-
tigen binding molecules were subjected to five rounds of selection, using decreasing concentrations of bioti-
nylated extracellular domain of human HER2 (HER2-ECD) as
20 the target (recombinant human HER2 extracellular domain, amino acids 238-2109, provided by Fox Chase Cancer Cen-
ter, Philadelphia, USA)

80 and 260 colonies clones obtained from the fourth and fifth round, respectively, were picked in order to
25 perform an analysis of their HER2 binding activity.

ABAS ELISA for analysis of HER2 binding

Clones randomly chosen from the fourth and fifth round of the selection were produced in 96 well plates
30 (Nunc). An ELISA screening procedure, termed ABAS ELISA, was used to identify high affinity HER2 binding Z vari-
ants. Single colonies were inoculated in 1 ml TSB-YE me-
dium (30.0 g Tryptic Soy Broth (Merck) and 5.0 g Yeast extract (Merck), water to a final volume of 1 l) supple-
35 mented with 1 mM IPTG and 100 μg/ml ampicillin in deep well 96 well plates and grown on a shaker over night at 37 °C. Cells were pelleted by centrifugation at 3000 *g*

for 10 minutes. The pellets were resuspended in 300 µl
PBS-T and frozen at least for 30 minutes at -80 °C. The
plates were thawed in tepid water and centrifuged at 3500
g for 20 minutes. 100 µl of the supernatants were loaded

5   on microtiter wells (#9018 Costar®) which had been incu-
bated with 6 µg/ml human serum albumin (HSA) in 15 mM
Na_2CO_3 and 35 mM $NaHCO_3$ (pH 9.6) over night at 4 °C and
blocked with 2 % skim milk powder in PBS-T for 1 h at
room temperature. The plates were washed four times,

10 prior to addition of 100 µl 1 µg/ml biotinylated HER2 per
well and incubated for 1.5 h. After washing the wells
four times, 100 µl Streptavidin-HRP (1:5000) (#P0397
Dako) per well were added and incubated for 1 h. The
wells were washed four times and after the final wash,

15 100 µl developing solution (ImmunoPure) TMB (#34021
Pierce) was added to each well. After 20-30 minutes, 100
µl stop solution (2 M H_2SO_4) was added to each well. The
absorbance at 450 nm was measured in an ELISA reader
(Tecan). The results from the ABAS ELISA is presented in

20 Figure 29. The X axis of the diagrams of Figure 29A and
29B correspond to the numbering of the well in the 96
well plate in question – thus, Figure 29A shows the ELISA
results for a first 96 well plate, whereas Figure 29B
shows the results for a second plate.

25

DNA sequence analysis
      Sequencing of DNA encoding the Z_{HER2} variants ana-
lyzed in the ABAS-ELISA was performed with ABI PRISM®
dGTP, BigDye™ Terminator v3.0 Ready Reaction Cycle Se-

30 quencing Kit (Applied Biosystems) according to the manu-
facturer's recommendations, using the biotinylated oli-
gonucleotide AFFI-72 (5'-biotin-CGGAACCAGAGCCACCACCGG).
The sequences were analyzed on an ABI PRISM® 3100 Genetic
Analyser (Applied Biosystems). The sequence analysis re-

35 sulted in 130 unique sequences. Sequences originating
from Z_{HER2} variants that exhibited binding in the ABAS
ELISA experiment, as evidenced by an absorbance value

that is at least two times higher than the value of the
negative control, are presented in Figure 1 and identi-
fied in the sequence listing as SEQ ID NO:6-76. The no-
menclature of these HER2 binding Z variants is as fol-
5   lows. The variants isolated from the first plate of the
ELISA experiment (Figure 29A) are denoted $Z_{HER2:1NN}$, where
NN corresponds to the number of the well in the first
plate in which that particular polypeptide variant was
analyzed. The variants isolated from the second plate of
10   the ELISA experiment (Figure 29B) are denoted $Z_{HER2:2NN}$,
where NN corresponds to the number of the well in the
second plate in which that particular polypeptide variant
was analyzed.

15   *Cloning and protein production*

Selected HER2 binding polypeptides were expressed in
E. coli cells using an expression vector encoding con-
structs that have been schematically illustrated in Fig-
ure 30. The polypeptides were produced as fusions to an
20   N-terminal hexahistidyltag. The His_6-tagged polypeptides
$Z_{HER2:101}$, $Z_{HER2:107}$, $Z_{HER2:149}$, $Z_{HER2:202}$, $Z_{HER2:205}$, $Z_{HER2:207}$, $Z_{HER2:209}$,
$Z_{HER2:222}$, $Z_{HER2:225}$ and $Z_{HER2:229}$ as well as $Z_{HER2\ A}$ were purified
using a BioRobot 3000 (Qiagen) and the NI-NTA Superflow
96 BioRobot procedure under denaturating conditions. Pu-
25   rified, His_6-tagged proteins were dialyzed in PBS (2.68
mM KCl, 137 mM NaCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH
7.4). The protein concentration of samples was calculated
from the measured absorption value at 280 nm and the
theoretical extinction coefficient of the respective pro-
30   tein.

Biosensor analysis of the His_6-tagged Z_{HER2} variants

The interactions between the His-tagged Z_{HER2} vari-
ants produced according to the preceding section and HER2
35   were analyzed using surface plasmon resonance in a
Biacore® 2000 system (Biacore AB, Uppsala, Sweden). Human
HER2 and IgG (immunoglobulin G) were immobilized in dif-

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ferent flow cells by amine coupling onto the carboxylated
dextran layer on surfaces of CM-5 chips, according to the
manufacturer's recommendations. Immobilization of human
HER2 and IgG resulted in 4600 and 5100 resonance units
5   (RU), respectively. A third flow cell surface was acti-
vated and deactivated for use as blank during injections.
The fusion polypeptides $Z_{HER2:101}$, $Z_{HER2:107}$, $Z_{HER2:149}$, $Z_{HER2:202}$,
$Z_{HER2:205}$, $Z_{HER2:207}$, $Z_{HER2:209}$, $Z_{HER2:222}$, $Z_{HER2:225}$ and $Z_{HER2:229}$ were
diluted in 1x HBS-EP (5 mM HEPES, 150 mM NaCl, 3.4 mM
10   EDTA, 0.005 % surfactant P-20, pH 7.4) to a final concen-
tration of 50 nM, and injected at a constant flow rate of
10 μl/minute. The total injection time was 2 minutes (as-
sociation) followed by a wash during 3 minutes (dissocia-
tion). The surfaces were regenerated with two injections
15   of 25 mM HCl (30 seconds/injection). The responses meas-
ured in reference cells (activated/deactivated surface)
were subtracted from the response measured in the cells
with immobilized HER2. The ability of the purified pro-
teins to interact with HER2 was confirmed, as illustrated
20   by the sensograms of Figure 31.

<u>Example 10</u>
<u>Identification and characterization of additional HER2-
binding polypeptides</u>
25

A comprehensive sequence analysis was performed of
clones obtained after the third and fourth round from the
selection described in Example 1. DNA was sequenced using
the ABI PRISM® dGTP, BigDye™ Terminator v3.0 Ready Reac-
30   tion Cycle Sequencing Kit (Applied Biosystems) according
to the manufacturer's recommendations and an ABI PRISM®
3100 Genetic Analyser (Applied Biosystems). The bioti-
nylated oligonucleotide AFFI-72 (5'-biotin-
CGGAACCAGAGCCACCACCGG) was used as a primer. Sequence
35   analysis revealed 11 new polypeptide sequences, i e
clones that had not been found in the study described in
Example 1.

It was decided to express these Z variants in *E. coli* cells, using an expression vector encoding constructs that are schematically illustrated in Figure 30. The polypeptides were thereby produced as fusions to an

5   N-terminal hexahistidyl tag. The polypeptides were purified by Immobilized Metal ion Affinity Chromatography (IMAC) using a BioRobot 3000 (Qiagen) and the NI-NTA Superflow 96 BioRobot procedure under denaturing conditions. The eluted proteins were analyzed on SDS-PAGE. The

10   buffers of purified His_6-tagged proteins were exchanged to 5 mM NH_4Ac using PD-10 columns (Amersham Biosciences) according to the manufacturer's recommendations. Thereafter, the proteins were lyophilized and dissolved in HBS-EP (5 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005 % surfac-

15   tant P-20, pH 7.4). Protein concentrations of samples were calculated from the measured absorption values at 280 nm and the theoretical extinction coefficient of the respective protein.

20   *Biosensor analysis of the His_6-tagged Z_{HER2} variants*

     The binding activity between the His_6-tagged Z_{HER2} variants and HER2 was analyzed using a Biacore® 2000 (Biacore AB, Uppsala, Sweden). Human HER2 and HIV-1 gp120 (Protein Sciences Corporation, #2003-MN) were immobilized

25   in different flow cells by amine coupling onto the carboxylated dextran layer on surfaces of CM-5 chips, according to the manufacturer's recommendations. Immobilization of human HER2 and HIV-1 gp120 resulted in 2631 and 3138 resonance units (RU), respectively. A third flow

30   cell surface was activated and deactivated for use as blank during injections. The Z_{HER2} variants were diluted in 1x HBS-EP (5 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005 % surfactant P-20, pH 7.4) to a final concentration of 1 μM, and injected at a constant flow rate of 10

35   μl/minute. The total injection time was 1 minute (association) followed by a wash during 3 minutes (dissociation). The surfaces were regenerated with an injection of

10 mM HCl during 30 seconds. The responses measured in
reference cells (activated/deactivated surface) were sub-
tracted from the response measured in the cells with im-
mobilized HER2. Three of the purified proteins, $Z_{HER2:3053}$

5    $Z_{HER2:0434}$ and $Z_{HER2:0024*}$, bound specifically to HER2 as il-
lustrated by the sensorgrams of Figure 32. The sequences
of these Z_{HER2} variants are presented in Figure 1, and
identified in the sequence listing as SEQ ID NO:77-79.